Role of Lysosomes in Nonshivering Thermogenesis

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ABSTRACT

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Obesity occurs when nutrient intake exceeds energy expenditure over prolonged periods. In the modern world, obesity has reached epidemic proportions. Complications of obesity, including cardiovascular disease, non-alcoholic fatty liver disease, certain forms of cancer, and metabolic dysfunction contribute substantially to morbidity and death today. With 13% of the world's population affected, the rising rates of obesity will grow as a public health burden. Until recently, pharmacologic attempts to treat obesity have focused on reducing food intake. However, motivated in part by recent studies in mice and by analyses of fat in humans, approaches to increasing energy expenditure, specifically thermogenic energy expenditure, may provide a new therapeutic avenue.

Most simplistically, there are two classes of adipocytes: storage and thermogenic. Storage fat, typically composed of unilocular white adipocytes function as storage depots for excess calories. On the other hand, thermogenic fat containing brown or beige adipocytes, generate heat through uncoupled mitochondrial respiration, This regulated generation of heat, known as thermogenesis, is used by organisms to maintain or increase body temperature. Historically, thermogenesis has been divided into shivering and nonshivering thermogenesis. Repeated, rapid contraction of skeletal muscles generate heat and is the basis for shivering thermogenesis. Nonshivering thermogenesis (NST) describes all the other mechanisms by which an organism can generate regulated heat. Only two organelles are known to contribute to NST: the mitochondrion of brown and

beige adipocytes and the sarcoplasmic reticulum of muscle. The role of other organelles has not been systematically studied.

Here we show in mice that thermogenic stimuli, including a cold challenge and pyrogenic molecules, activate a lysosomal program in a known thermogenic tissue (BAT) as well as several "non-thermogenic" organs, including the spleen, liver and skeletal muscle. A similar program is activated by a cold challenge in the metazoan, *Drosophila melanogaster,* suggesting an evolutionarily ancient origin for this response. We show by both pharmacologic and genetic means that impairment of lysosomal function compromises the thermogenic response of individual cells *ex vivo* and of mice *in vivo*. Data from genetic manipulations find that impairment of lysosome function leads to cold intolerance and death as well as modestly downregulate the classical *Ucp1* thermogenic program. However, pharmacological inhibition reveals that impairment of lysosome function can compromise thermogenesis without altering the *Ucp1* program.

As part of our efforts to study lysosome function in thermogenesis, we developed a new method of measuring thermogenesis in primary cells. Using isothermal titration calorimetry (ITC), we quantitatively measured the heat generated by cells isolated from mice. This permitted us to assess the effects of both genetic and pharmacologic manipulations on the generation of heat and allowed us, for the first time, to measure the heat (uCal/sec/cell) of BAT in the basal and stimulated state. With ITC, we demonstrated that the impairment of lysosome function had direct effects on the generation of cellular heat, independent of systemic modulators of temperature such as basal metabolic rate or circulatory dissipation.

From these studies, we conclude that lysosomes are thermogenic organelles induced by cold and pyrogenic stimuli and contribute both directly and indirectly to thermogenesis. Our work also suggests that lysosome thermogenesis may provide a means of thermoregulation in poikilotherms as well as in tissues previously not implicated in temperature regulation in mammals.

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CHAPTER ONE

INTRODUCTION

The Obesity Epidemic

The arc of human health and epidemiology has been marked by periodic abrupt and dramatic shifts. Large environmental changes, mass migrations, and societal development skills such as agriculture and sewage systems, have rapidly changed the health of large populations. Complementarily, in the last century there has been an abrupt, precipitous decline in deaths from acute infectious diseases and a parallel rise in deaths from chronic disorders.

The discovery of antibiotics and antisera, provided a means to treat most bacterial and many viral infections. In parallel, as the twentieth century progressed, the hunger and malnutrition that had once been widespread became no longer in developed countries. Instead, the converse – an abundance of inexpensive food – became an unexpected source of a new epidemic. Ironically, our ingenious solutions to previous generations' problems have given rise to a new unexpected problem: a curse of abundance, manifested in biological form as the obesity pandemic.

In simplest terms, obesity can be reduced to a thermodynamic formula consisting of only two variables: energy intake and energy expenditure. When energy intake exceeds that of energy expenditure, the individual is in a state of negative energy balance, resulting in weight loss. However, when the latter exceeds that of the former, there is a state of positive energy balance, resulting in weight gain. The

chronic tipping of this energy scale towards weight gain can ultimately lead to obesity. Globalizing factors on culture and national developments have changed the feeding and exercise behaviors even in developing nations. Obesity is a public health challenge not only in the West, but also worldwide.

Unfortunately, obesity is more than just a concern of body aesthetics; it also significantly contributes to morbidity and mortality. Currently 78.4 million (Ogden et al. 2014) individuals in the United States alone and an additional 600 million worldwide ("WHO — Obesity and Overweight" 2016). Comorbidities of obesity include cardiovascular disease (Menke et al. 2007), diabetes (Ali H. Mokdad 2001)(A. H. Mokdad et al. 2000), non-alcoholic fatty liver disease (Peng et al. 2012; Watts et al. 2001; Chan et al. 2002), certain cancers (Richards and Lisa 2009; Magliano and Dianna 2013; Pendyala, Swaroop, and Peter 2008; Brown and Simpson 2013; L. Chen et al. 2016) and neurodegeneration (Sriram et al. 2002; Platt et al. 2016; Nuzzo et al. 2015; MacPherson et al. 2015; McGuire and Ishii 2016). Such consequences create a financial toll for both individuals and society at large. Therefore, while the obesity epidemic is easy to dismiss superficially, it has enormous and devastating consequences.

One potential therapeutic avenue for the treatment of obesity is through activation brown adipose tissue (BAT). Brown fat and its functionally related beige congener are catabolic organs that can burn calories through heat production. Therefore, BAT has the potential to increase energy expenditure and alter the thermogenic equation in favor of weight loss. This possibility has led some to imagine that BAT driven energy expenditure can provide a therapeutic opportunity for treating or preventing obesity. The majority of research into brown adipose tissue has focused on its transcriptional regulation and less on its

cell biology and molecular mechanisms, particularly of heat generation. This dissertation explores some aspects of the cellular and molecular mechanisms of heat generation by examining an organelle that has never been associated with thermogenesis until just recently (Martinez-Lopez et al. 2016). Our studies of the lysosome reveal a critical role for its function in homeothermy. Our findings offer potential new avenues for understanding brown adipose tissue and new approaches to develop therapies for obesity and related metabolic diseases.

Therapeutic Interventions

The remarkably high prevalence of obesity worldwide provides a strong incentive to find viable treatments. Pharmaceuticals have invested billions in obesity research to date. A large portion of the budget of NIH NIDDK funds the field. Yet, even in the face of such financial and temporal investment, outside of the invasive and expensive bariatric surgical options (Wolfe, Kvach, and Eckel 2016), simple, safe, and viable treatments still elude us, making obesity one of the most notoriously complex and difficult disease models to study and to treat.

The comorbidities of obesity are numerous; as a result, obese patients are frequently placed under strict pharmacological regimens, either prophylactically or correctively. Nonetheless, this treatment philosophy inherently only targets the peripheral consequences of extraordinary weight gain and not the phenomenon itself. This unfortunate reality is due in part to the lack of effective prescription drugs for weight loss. Current safe options generally target energy intake and allow patients to lose on average, an unsatisfying 5-10% of their body weight over one year when used in conjunction with difficult lifestyle interventions such as diet and exercise (Nicklas et al. 2012); (Wing and Phelan 2005; Dombrowski et al. 2014). Even with modest changes in weight loss, side effects from such drugs

often make compliance difficult. Orlistat, an over-the-counter drug that prevents lipid absorption, results in uncomfortable and socially awkward digestive side effects(Cavaliere, Floriano, and Medeiros-Neto 2001). Appetite suppressants such as phentermine or desoxgen, methamphetamines, alter the central nervous system and result in sympathetic effects that exacerbate cardiovascular disease risk (Yosefy, Chaim, and Ronen 2010; Marks et al. 2016; Paratz, Cunningham, and MacIsaac 2016). Ultimately, these pharmacotherapies target only one variable of the energy homeostasis equation: energy intake, a characteristic clearly unfeasible for longterm effective weightloss.

In the most dire cases, bariatric surgery becomes a potential option for patients. This option is often considered to be of last resort, only for patients with morbid obesity (BMI ≥ 40) who fail to lose weight even after strict dieting, exercise, and pharmacological support (Wolfe, Kvach, and Eckel 2016). Bariatric surgery comes in several forms: gastric band, gastric sleeve, roux-en-y gastric bypass, and biliopancreatic diversion with duodenal switch (J. Lee et al. 2016)(Hess 2007). Given the invasive nature of these surgeries, the usual surgical risks are involved; the estimated mortality post surgery is around 0.5-2%(Hess 2007; Flum and Patchen Dellinger 2004), alarmingly high for a preventable disorder. Nutritional deprivation and inadequacies are frequent among postoperative patients (Gletsu-Miller and Wright 2013; O'Donnell and Kelly 2006; Shankar, Boylan, and Sriram 2010). Sudden changes in diet and nutrition also lead to increased risk for bone loss (Casagrande et al. 2012; Lemke et al. 2013). Long term weight loss from bariatric surgery ranges from 15-40% from total body weight, indicating its efficacy (Mahawar et al. 2015; Topart et al. 2012; Courcoulas et al. 2013). Nonetheless, given the previously enumerated risks as well as prerequisites for candidacy, bariatric surgery is not a viable option for all obese patients.

Therefore, increasing energy expenditure safely via a pill, colloquially known as the diet pill, would be the holy grail for metabolic interventions. Currently, patients can address this variable through exercise, but maintaining compliance represents a substantial challenge. The discovery of active brown adipose tissue in humans (Jan Nedergaard, Bengtsson, and Cannon 2007; Saito et al. 2009; Virtanen et al. 2009; van Marken Lichtenbelt et al. 2009) has provided a new strategy to develop anti-obesity pharmacotherapies. Nonetheless, the amount of brown adipose tissue in humans, inducible or not, can be so marginal that the amount it can contribute to energy expenditure is dubious. This dissertation proposes a novel way of increasing energy expenditure that can target cell types beyond the brown adipocyte.

Thermodynamics

While the clinical and biomedical implications of this dissertation applies to obesity and metabolism, it is ultimately on the study of cellular thermogenic regulations and principles. As the focus of thermodynamics is on energy and matter transfer, the field provides the physical properties that explain metabolic phenomena. Therefore, thermogenesis and its metabolic benefits ultimately relies on the basic principles of thermodynamics, particularly the laws that govern biological systems.

General Overview

Thermodynamics is the study of energy transfer in relation to a system. Historically, the term was coined around 1840 from the Greek words *therme*, "heat," and *dynamis*, "power." Likewise, for most of its research lifetime, the field focused on the effects of heat and work due to its relationship with the

development of engines at the time. A macroscopic science, thermodynamics focuses only on a few variables and does not concern itself with microscopic behaviors of its components.

In thermodynamics, there are several key definitions and physical entities: system, state, equilibrium, and process. The "system" is a finite, bounded quantity of matter, and everything exterior of the boundary is known as the "surroundings." A closed system is a specific class of system where matter is incapable of crossing the boundary, allowing for conservation of mass to be satisfied. The "state" of the system is a set of measurable properties defined when a system is in equilibrium. Therefore, when the state of a system is unchanged, then the system is in equilibrium. Equilibrium can be mechanical, thermal, and chemical. Finally, when the equilibrium is disrupted and the state of the system changes, then a "process" is occurring. All processes are irreversible to some degree, a phenomenon known as inefficiency.

Laws of thermodynamics

In thermodynamics, there are four defining laws that characterize all thermodynamic systems.

Zeroth law of thermodynamics

The zeroth law of thermodynamics states that if two distinct systems are in thermal equilibrium with a third distinct system, than the two systems are also in thermal equilibrium with each other. With this characterization, the law allows for for an equivalence relation and was expressed in more physically meaningful terms by Maxwell: "All heat is of the same kind"

First law of thermodynamics

The first law of thermodynamics, also known as the law of energy conservation, states that changes in the internal energy of a closed system must be equal to the difference between the energy applied and performed by the system. Therefore, a perpetual motion machine is impossible. Further, the law of conservation of energy suggests that energy can neither be created nor destroyed, but can change forms, the fundamental concept behind uncoupled thermogenesis by UCP1.

Second law of thermodynamics

The second law of thermodynamics is an empirical principle asserting that entropy is always positive. As a result, all processes must be irreversible to some extent as energy will dissipate.

The conception of the second law came out of disparities between the theoretical implications of the first law and actual observed thermodynamic events. While the first law provides symmetry between the initial and final states of an evolving system, it fails to explain how natural processes seem to prefer a specific state. By establishing the notion of "entropy," the second law allows the final state to be of lower energy, and hence preferred, over the initial state of higher energy. The second law does not violate the first law, as the differential energy between the initial and final states has simply dissipated. As such, the overall energy of the system still conserved.

Third law of thermodynamics

The final and third law of thermodynamics argues that the entropy of a system at absolute zero is a constant. This law allows for a reference point for entropy and known as the Boltzmann's constant.

Biological thermodynamics

Energy is required for all life and serves as the fundamental basis for chemical reactions, and hence biological reactions. The core principles of thermodynamics in biological systems that will be discussed in this chapter rely primarily on the first and second laws of thermodynamics.

Biological thermodynamics study primarily the various biological dynamics within the organisms and cells. Therefore, the applicability of thermodynamics extend far beyond that of its original purpose for engines and machinery. As stated by Einstein in his autobiography:

> "A theory is the more impressive the greater the simplicity of its premises is, the more different kinds of things it relates, and the more extended its area of applicability. Therefore the deep impression which classical thermodynamics made upon me. It is the only physical theory of universal content concerning which I am convinced that, within the framework of the applicability of its basic concepts, it will never be overthrown."

Indeed, this framework is of particular importance to this dissertation as it attempts to use its foundations to describe a novel feature of lysosomal biology as well as the regulation of the heat generating phenomenon, thermogenesis, in homeotherms.

Brown Adipose Tissue

The brown adipocyte: the only known cell devoted to regulating the host organism's body heat, a necessity for and unique quality of homeotherms. A century ago, this tissue was oddly known as the "hibernation gland;" today, it is lauded as the "good fat," creating a false dichotomy of physiological values on fat depots when in fact the lack of "bad" white adipose tissue leads to profound metabolic disturbances in lipid and glucose homeostasis.

The evolution of our knowledge of the brown adipocyte has progressed at a slow pace. While the tissue was first discovered in 1551 by German physician and botanist Konrad Gesner (Gessner, Conrad, and Burndy 1551), it took another 400 years before BAT was dubbed the "hibernation gland" (Sheldon 1924) for its role in torpor, a physiological state of reduced body temperature and metabolic rate (Davenport and John 1992; Staples 2016). An additional forty years proceeded before researchers postulated that the tissue produced heat (Smith and Hock 1963; Cameron and Smith 1964; Smith 1964). Nearly twenty years later, the uncoupling of mitochondrial H^+ gradient by the Uncoupling Protein 1 (UCP1) was at last identified, cloned, and suggested to be responsible for thermogenic ability of brown adipocytes (Nicholls, Bernson, and Heaton 1978). The study of brown adipocytes remained an area of active research but were assumed to be irrelevant to adult humans (Jan Nedergaard, Bengtsson, and Cannon 2007). However, about a decade ago, a series of studies (Christensen, Clark, and Morton 2006; Garcia et al. 2006; Garcia et al. 2004; Heiba et al. 2005; Jacobsson et al. 2005; Sturkenboom et al. 2004; Virtanen et al. 2009; van Marken Lichtenbelt et al. 2009; Saito et al. 2009) showed that brown adipocytes indeed exist in adult humans. This revelation was then supported by the discovery of

the "beige/brite" adipocyte, an inducible thermogenic cell similar to the brown adipocyte in function (Seale et al. 2008; S. Cinti 2002; Himms-Hagen et al. 2000; Wu et al. 2012; Petrovic et al. 2010) that exists in humans (Bartesaghi et al. 2015; P. Lee et al. 2014; Lidell et al. 2013). For many, these discoveries unleashed a fervor of excitement - activation of these thermogenic adipocytes to alter thermodynamic balance could induce weight loss and allow for a viable anti-obesity therapy.

Thermogenesis

While energy is essential for life and is often in limited supply, reducing its efficiency may be beneficial in a limited set of circumstances. Biological systems function in a specific temperature range beyond which life becomes impossible and in a narrower range in which biological reactions are most efficient. Organisms maintain this temperature range compatible with both life and optimal biological function through heat generation and exchange, preventing the hazardous effects of large environmental temperature variations. Although ectotherms rely primarily on external sources of heat, endotherms draw on internal sources - primarily, thermogenesis. This latter phenomenon, thermogenesis, is the core of brown adipose tissue biology.

Most biological reactions generate small amounts of heat due to inherent inefficiencies as a result of second law of thermodynamics. So called biological "futile cycles" take advantage of this, by undergoing chemical transformations that end in the same chemical states they began (Qian and Beard 2006). Such processes therefore dissipate enthalpy and generate heat, contributing to the overall thermogenic output of an organism (Tseng et al. 2010; Rolfe and Brown

1997). There are several known examples of regulated futile cycles: transmembrane ion leaks, muscle/actin-myosin relaxation through SERCA, and triglyceride/fatty-acid cycling (Tseng et al. 2010). The best studied regulated thermogenic process is the "uncoupling" of the inner mitochondrial membrane through the actions of Uncoupling Protein 1 (UCP1), a phenomenon usually categorized as "nonshivering" thermogenesis (NST).

UCP1, a small protein of 32 kDa, is unique to thermogenic adipocytes and is dependent on mitochondrial biology. The protein is expressed exclusively in the inner mitochondrial membrane where substrates such as ADP, ATP, phosphate, oxoglutarate, citrate, glutamate, and malate are transported (Wohlrab and Hartmut 2009; Krämer and Klingenberg 1977). The inner membrane is the site for ATP production by oxidative phosphorylation (Berg, Tymoczko, and Stryer 2002). The endergonic production of useful chemical energy is coupled to the exergonic process of electron flow generated by the electron transport chain. This flow of electrons creates an uneven distribution of H^+ protons in the intermembrane space, thereby generating an electrochemical gradient (Berg, Tymoczko, and Stryer 2002). Chemiosis requires the protons to move down its electrochemical gradient across the inner membrane (Mitchell and Moyle 1967). In the mitochondria of most cells, chemiosis can only occur through ATP-synthase, where ATP is generated when the protons flow into the mitochondrial matrix (Berg, Tymoczko, and Stryer 2002; Schultz and Chan 2001).

UCP1 functions by disrupting the transmembranal electrochemical gradient established by the mitochondrial electron transport chain (Nicholls, Bernson, and Heaton 1978). As a result, the protons in the intermembrane space can bypass the ATPase pump and flow down the gradient without synthesizing ATP.

Energy that would have been converted to useful chemical energy, or ATP, is instead dissipated as heat, as dictated by first law of thermodynamics (Nicholls and Locke 1984). As a proton transporter, UCP1 shares a similar tripartite structure with other mitochondrial carriers, but has unique matrix facing and C-terminus sites that likely confer UCP1's function (Klingenberg, Martin, and Shu-Gui 1999; J. Nedergaard et al. 2001). While UCP1 is part of a larger UCP family that also includes both UCP2 and UCP3, presence of UCP1 is necessary for this uncoupling mechanism; neither UCP2 nor UCP3 are capable of the uncoupling mechanism of UCP1 (Matthias et al. 2000).

While the brown adipocyte is not unique in its thermogenic abilities, its expression of UCP1 allows it to be only known cell devoted to heat generation and is central to nonshivering thermogenesis. NST is the most well known mammalian process that creates heat to maintain body temperature in the face of a cold challenge.

During cold exposure, temperature sensitive neurons in the skin (McKemy, Neuhausser, and Julius 2002; Peier et al. 2002; Bautista et al. 2007; H. Lee et al. 2005; Moqrich et al. 2005) as well as peripheral (Riedel 1976; Gupta, Nier, and Hensel 1979) and central thermoreceptors (Guieu and Hardy 1970; Tominaga et al. 1998; Bautista et al. 2007) on internal organs signal to the hypothalamus, activating the adrenal glands to secrete norepinephrine and epinephrine(Caldwell, Graves, and Wallace 1995). Simultaneously, these catecholamines are released locally by the neurons innervating the brown adipose tissue (Wirsén 2011; Bargmann, von Hehn, and Lindner 1968; Murano et al. 2009). The β-adrenergic receptors and α-1-adrenergic receptor on the membrane of brown adipocytes are

thereby activated (Svartengren 1982; Kurahashi, Masashi, and Akihiro 1979; Horwitz and Hamilton 1984). These adrenergic receptors are coupled to G_s-proteins and activate adenylate cyclase upon stimulation(Gal et al. 1984; Levitzki and Alexander 1976; Milde et al. 2014). The resultant increase in cyclic adenosine monophosphate (cAMP) levels by adenylate cyclase activates protein kinase A (PKA). PKA then proceeds to promote NST through three signaling pathways: perilipin (PLIN1), p38 mitogen-activated protein kinase (p38 MAPK), and cAMP response element binding-protein (CREB) (Collins 2011; Cao et al. 2004; Bartelt and Heeren 2012). CREB and phospho-p38 MAPK work together to generate UCP1 production and action in the mitochondria. Together, they activate the transcriptional machinery including transcription factor 2 (ATF2)(Cao et al. 2004), zinc finger protein 516 (ZFP516)(Dempersmier et al. 2015), peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC1α)(Cao et al. 2004), PR domain containing 16 (PRDM16)(Dempersmier et al. 2015), peroxisome proliferator-activated receptor α (PPARα), peroxisome proliferator-activated receptor gamma (PPARgamma) and the retinoid x receptor (RXR)(Collins 2011; Bartelt and Heeren 2012). Once formed, the transcriptional complex drives factors that activate UCP1 transcription, translation, and insertion into the mitochondrial inner membrane (Figure 1.1)(Yubero et al. 1998; Hondares et al. 2011; Barbera et al. 2000; G. Zhang, Sun, and Liu 2016).

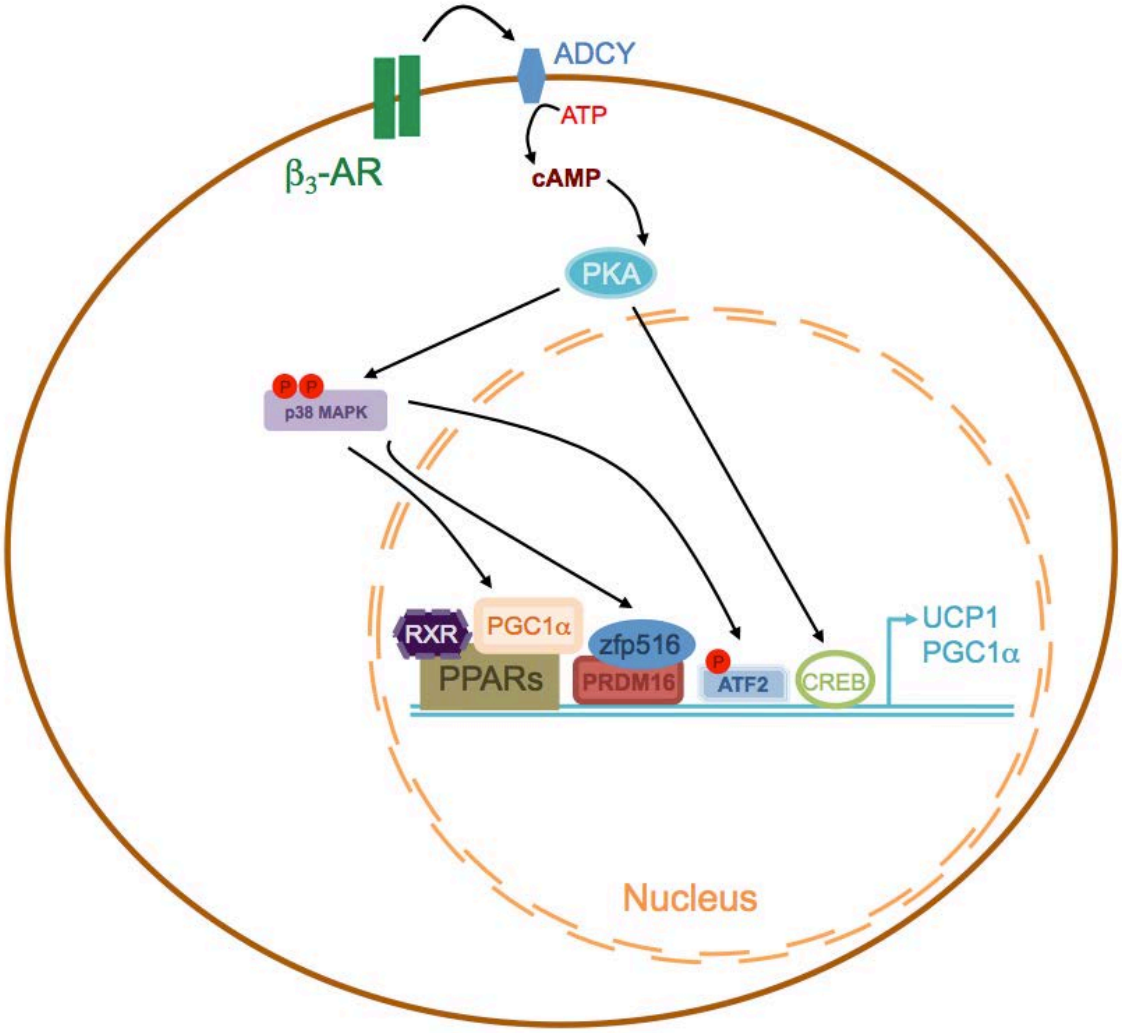

Figure 1.1 : Nonshivering Thermogenesis Signaling Cascade in the Brown Adipocyte

This same PKA dependent signaling cascade also induces perilipin dependent activation of neutral lipolysis in adipocytes, generating fatty acids – potential

substrates for UCP1 uncoupled oxidation (Miyoshi et al. 2006; Granneman et al. 2009; Subramanian et al. 2004). It has been assumed that lipid stored in brown adipocytes then serves as a primary substrate for uncoupled thermogenesis (Calderon-Dominguez et al. 2015).

Fatty acids and Thermogenesis

Fatty acids are carboxylic acids with an aliphatic chain of varying lengths; these chains confer unique cellular properties. When metabolized via beta oxidation, fatty acids, per mole, yield as much as 129 ATP for the cell. Consequently, oxidation of fatty acids become the main source of "fuel" for thermogenesis. There are four different sources of fatty acids: 1) *De novo* synthesis by fatty acid synthetase, 2) localized hydrolysis of lipoproteins by extracellular lipase, i.e., lipoprotein lipase, 3) free fatty acid uptake from the circulation, and finally 4) lipolysis of intracellular lipid droplets by neutral lipases (Calderon-Dominguez et al. 2015). Current dogma suggests that lipolysis of lipid droplet triglycerides via adipose triglyceride lipase (ATGL/PNPLA2/Desnutrin), and regulated by adrenergic pathways is the primary source of fuel for brown adipocyte thermogenesis (Morak et al. 2012; Ahmadian et al. 2011; Cannon and Nedergaard 2004). The initial studies I undertook were designed to test whether the neutral lipase ATGL is necessary for NST, and whether acid lipolysis via lysosomal acid lipase (LIPA) could play a significant role.

Fatty acids, however, serve as more than just fuel for mitochondrial oxidation. Both fatty acids (Shabalina et al. 2008) and purine nucleotides (Rial and González-Barroso 2001; Shabalina et al. 2004) interact with UCP1 to enhance its uncoupling capabilities. Several laboratories have proposed a number of potential mechanisms. One model posits that the fatty acid localizes to binding sites in the

proton conduction channel of UCP1, where their acidic moeities serve as a bridge for protons to pass through the membrane (Winkler and Klingenberg 1994). Another potential mechanism imagines fatty acids as proton shuttles, with the UCP1 protein catalyzing the necessary steps (Garlid, Jabůrek, and Jezek 1998; Fedorenko, Lishko, and Kirichok 2012). Fatty acids have also been conjectured to compete with purine nucleotides at either an activating or allosteric site on UCP1, thereby altering its protonophoric properties via structural changes (Rial and González-Barroso 2001).

Although the mechanism by which fatty acids facilitate UCP1-dependent thermogenesis is unclear, these findings point to fatty acids as signaling molecules in addition to substrates for NST.

Cellular Biology of Brown Adipose Tissue

Brown adipocytes differ from their white counterparts in several qualities other than their expression of UCP1. The defining features of brown versus white adipocytes are lipid droplet morphology and mitochondrial number. White adipocytes contain unilocular droplets that occupy over 90% of the volume of each cell, pushing other organelles to the plasma membrane. Brown adipocytes, on the other hand, have multilocular lipid droplets surrounded by large numbers of mitochondria that give brown adipocyte their eponymous color. Additionally, BAT depots, compared to white adipose tissue (WAT) depots, are highly innervated and vascularized, characteristics that can facilitate thermogenesis (Harms and Seale 2013; Park, Kim, and Bae 2014; Saely, Geiger, and Drexel 2012).

BAT depots are also located in anatomically distinct locations: WAT depots are found under the dermis (subcutaneous)(Saverio Cinti and Saverio 2001; S. Cinti 2012), surrounding visceral organs regions (visceral)(Saverio Cinti and Saverio 2001; S. Cinti 2012), between muscle fibers (muscular) (Gallagher et al. 2005), and around blood vessels (vascular) (Gu and Xu 2013); classical BAT areas are located in the interscapular, cervical, periaortic sites in rodents and may be found in cervical-supraclavicular, periaortic, and perirenal regions in humans - although these regions are vary among individuals and age (Sacks and Symonds 2013; Cypess et al. 2015; Cypess and Kahn 2010; Sidossis and Kajimura 2015).

In the last decade, it has become apparent that a separate and distinct class of thermogenic adipocytes exists and is developmentally and anatomically distinct from classical brown adipocytes (Wu et al. 2012). These "beige" (also called "brite") thermogenic adipocytes are found within WAT depots and detectable after activation by cold exposure or catecholamine induction (Wu, Cohen, and Spiegelman 2013). Beige adipocytes have a remarkable transformative ability: At thermoneutrality, they express little UCP1, relatively few mitochondria, and contain unilocular lipid droplets; following adrenergic stimulation, these cells increase UCP1 expression, mitochondrial numbers, and thermogenesis(Wu, Cohen, and Spiegelman 2013). As a consequence, these adipocytes seem to exhibit intermediate features, such as paucilocular droplets and a few mitochondria, giving the adipocytes the name "beige" as a description of the color between white and brown (Wu, Cohen, and Spiegelman 2013; Giralt and Villarroya 2013; S. Cinti 2012).

Classical brown adipocytes are more closely related to muscle cells (myocytes), than to beige or white adipocytes, while beige adipocytes are more closely related

to white adipocytes than brown adipocytes. Both myocytes and brown adipocytes are derived from myogenic factor 5 (myf5) expressing precursors, while white and beige adipocytes, from myf5 non-expressing cells (Sanchez-Gurmaches et al. 2012; Seale et al. 2008). This surprising distinction between not only brown and white adipocytes, but also two supposedly functionally similar cells, brown and beige adipocytes, extends beyond their ontogeny. Both thermogenic adipocytes exhibit their own individual transcriptional profiles and gene signatures (Sanchez-Gurmaches and Guertin 2014). Using data profiling of these two cell types, researchers have only begun to examine the types of thermogenic cells in humans in order to understand their therapeutic utility (Seale, Kajimura, and Spiegelman 2009).

Therapeutic Utility of Brown Adipocytes

Initial demonstrations of BAT existence in humans were done via 18-fluoro-deoxyglucose uptake assays, detected by positron emission tomography/computed tomography (PET/CT) in individuals after cold exposure (Virtanen et al. 2009; Saito et al. 2009; Zingaretti et al. 2009; van Marken Lichtenbelt et al. 2009; Cypess and Kahn 2010). These demonstrations had scientists scrambling to elucidate the mysteries of thermogenic adipocytes in attempts to find a viable anti-obesity therapy.

More significantly however, human BAT is metabolically active and may contribute a significant portion to overall energy expenditure when activated. Ouellet, et. al., showed that a short three hour cold exposure led to an increase of 80% in basal metabolic rates, partly as a consequence of BAT activation (Ouellet, Véronique, et al. 2012). Further, this activation may increase lipid uptake from lipolysis and lipoproteins, potentially improving overall metabolic

profiles in the long term. Along the same lines, human data support a correlation between UCP1 expression and circulating serum TG and HDL (Chechi et al. 2013). Such observations support the potential therapeutic power of thermogenic adipocyte activation.

Nonetheless, new findings show that this optimism may be misplaced. Although it is clear that BAT in humans exist and may even contribute to basal metabolism when activated, the extent in which we can utilize BAT's energy expending abilities as a workable therapy for treating obesity is a question yet to be answered. There are many reasons to doubt its utility: calculations attempting to determine the contribution of BAT to weight loss estimate that 50 grams or more of active BAT is necessary to burn a modest 125 kcal/day in humans (Rothwell and Stock 1979). Additional studies have further supported this calculation, with estimates as low as 8 kcal/day (Ouellet, Labbé, et al. 2012). Even more discouragingly, given the tight central control over energy homeostasis in the body, it seems that an increase in expenditure by BAT would only be counteracted by orexigenic behavior initiated by the brain (Tam, Lecoultre, and Ravussin 2012). Nonetheless, researchers are still hopeful that BAT could be a weight loss maintenance option at minimum.

Introduction to Lysosomes

Discovered serendipitously in 1955 by Christian de Duve (de Duve et al. 1955), the Belgian cytologist, lysosomes have often been relegated to the undignified role as the "garbage disposal of the cell". De Duve himself described the lysosome as "a saclike structure surrounded by a membrane and containing acid phosphatase." Furthering the imagery, he christened his new discovery with the

name, “lysosome,” a portmanteau of the Greek words, *lysis*, “to loosen,” and *soma*, “the body.”(de Duve et al. 1955; Castro-Obregón 2010; Nicholls, Bernson, and Heaton 1978) The connotations stuck, for decades the lysosome was thought of nothing more than a vesicle of digestive enzymes. This simplistic, unglorified view was only recently given way as new evidence emerged that paints the lysosome as a dynamic organelle that is more than a digestive sac. Rather, it is major regulatory body critical to multiple cellular functions and a key the cell’s fate (Carmine Settembre, Carmine, Alessandro, et al. 2013).

Lysosome Structure

Like all cellular vesicles, the lysosome is a single lipid bilayer membrane bound organelle. However, this generalized description far from captures the nuanced complexity of this critical organelle. These complexities come in large part from the inherent degradative nature of the lysosome. One of the lysosomal membrane’s primary functions is to protect the cell from the lysosome’s acidic contents. A thick polysaccharide based coating, known as a glycocalyx, coats the lumenal side of the lysosomal membrane(Carroll and Mark 1989; Kolsen-Petersen 2015). This coating likely protects the membrane from degradation by the various hydrolases and low pH level (Carmine Settembre, Carmine, Alessandro, et al. 2013).

In addition to this protective component, an estimated 50 or more proteins reside on the lysosomal membrane, more than the average cellular vesicle (Schröder et al. 2007). The lysosomal membrane proteins available fall into six different functional categories: structural, ion channels, transporters, catabolic, trafficking,

and nutrient sensing (Callahan, Bagshaw, and Mahuran 2009; Schröder et al. 2007).

Lysosomes, along with late endosomes, maintain a low, acidic pH. The unusual pH of this organelle is maintained by the ion channel, chloride ion channel 7 (CLC7) (Graves et al. 2008; Kasper et al. 2005; Weinert et al. 2010) and the vacoular-type H^+-ATPase (V-ATPase) pump (Ohkuma, Moriyama, and Takano 1982), which pump chloride out of and protons into the lysosomal lumen, respectively. With these two proteins, along with the help of cation transporters mucolipin (MCOLN1/TRPML1) (F. Zhang et al. 2009; F. Zhang et al. 2011) and two pore calcium channels 1 and 2 (TPC1 and TPC2) (Calcraft et al. 2009), negative charges are moved out of the vesicle, while positive charges are moved in(Mindell 2012). The resultant acidic environment then becomes home to an estimated 60-70 known acid hydrolases. These degradative enzymes function best at a low pH and become significantly less active in the neutral pH of the cytosol. As a consequence, degradation of macromolecules by these powerful enzymes is limited within the lysosome.

The hydrolases are responsible for the degradative function of lysosomes. Among these proteins are glycosidases, lipases, nucleases, sulphatases, and peptidases, all necessary to breakdown the breadth of substrates: proteins, glycogen, lipids, and glycosaminoglycans (Carmine Settembre, Carmine, Alessandro, et al. 2013). In humans, functional mutations in any of these hydrolases result in some form of lysosomal storage disease each associated with characteristic phenotypes that sometimes result in premature morbidity (Vellodi 2005). The golgi apparatus modifies the enzymes with mannose-6-phosphate, allowing the enzymes to not only traffic to the lysosome, but also become secreted and taken up by other

cells, a complicating factor that needs to be considered when generating tissue specific animal models (Braulke, Thomas, and Bonifacino 2009; Ghosh, Dahms, and Kornfeld 2003). This phenomenon is the basis of enzyme replacement therapy for sufferers of lysosomal storage diseases (Neufeld 1980).

The defining function of lysosomes is the degradation of a wide variety of macromolecules. Unlike other degradative machinery in the cell (i.e., proteasomes), the lysosome degrade molecules and entire cellular structures, organelles, molecules derived from multiple cellular compartments, and even exogenously derived molecules (Ballabio 2016). Targeting of molecular and cellular components to lysosomes is tightly regulated and dependent on a large number of proteins. Nearly twenty various proteins have been implicated in these functions, mostly Soluble NSF Attachment Protein Receptor (SNARE) proteins (Pryor et al. 2004; Weber et al. 1998; Jahn and Scheller 2006) and Ras-related protein (RAB) GTPases (Zerial and McBride 2001; Rink et al. 2005; Ohya et al. 2009). RAB5 and RAB7 mediate the endolysosomal pathway, giving the cell the ability to take in various proteins and cellular substrates from outside the cell (Rojas et al. 2008; Wang et al. 2011; Zeigerer et al. 2012). SNAREs, in addition to driving endosome fusion with lysosomes, also engage in autophagosome-lysosome fusion required for functional autophagy (Itakura, Kishi-Itakura, and Mizushima 2012).

Trafficking events often result in intralysosomal vesicles which arise from lysosomal invagination. Degradation of the intralysosomal membranes is a curiosity as the lysosome must be able to selectively recognize intralysosomal membranes from the native lysosome membrane. These vesicles contain additional proteins that hydrolyze lipids and were discovered primarily through the study of defects in glycosphingolipid catabolism (Burkhardt et al. 1997).

Overview of Lysosomal Functions

The various lysosomal functions can be categorized into three groups in: (1) degradation, (2) secretion, and (3) signalling (Figure 1.2).

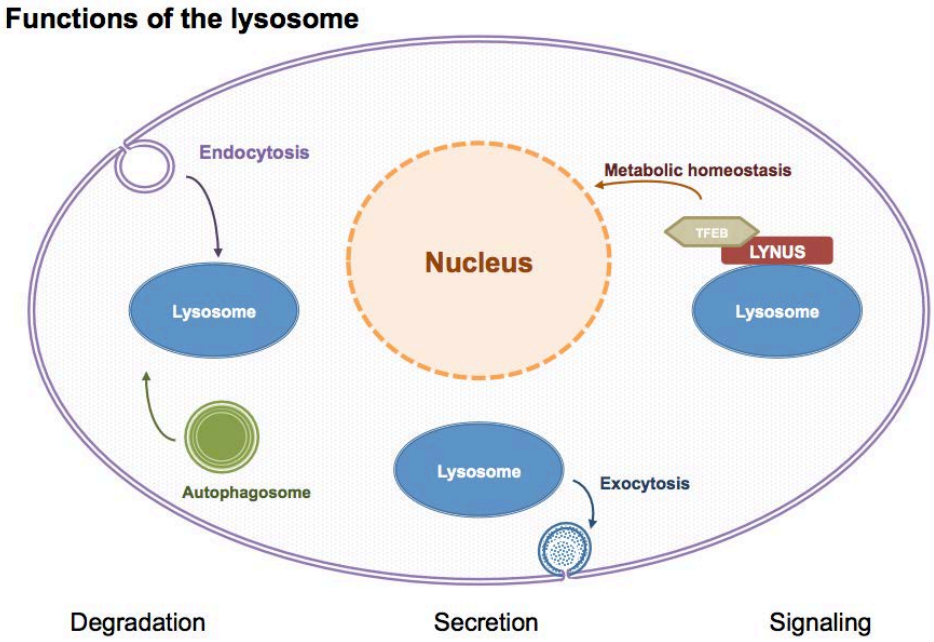

Figure 1.2: Overview of the various functions of the lysosome.

Degradation

As with any cellular process that requires trafficking, but particularly for an organelle that handles such large fluxes, the process requires precision in spite of its complexity. The eukaryotic cells have unique routes specific to the cargo type. Cargo can be both intra- and extracellular derived, the latter generally entering lysosomes through endocytosis. Endocytosis involves the creation of endosomes, which are among the main characters of lysosomal mediated degradations(J. P. Luzio et al. 2000). While endocytosis is often limited to receptor-mediated endocytosis, lysosomes are involved in the degradation of cargo from

phagocytosis, macropinocytosis, clathrin mediated endocytosis, and clathrin independent endocytosis (Conner and Schmid 2003; Doherty and McMahon 2009; Hansen and Nichols 2009; Sorkin and von Zastrow 2009).

Phagocytosis describes cellular phenomenon of vesicular internalization of solid particles, including bacteria. The immune system, particularly macrophages, use phagocytosis as part of an organism's immune defense strategy (Rosales 2008). When liquids are internalized, the process is known instead as macro/pinocytosis (LaBella 1973). Clathrin mediated endocytosis is the most understood route of endocytosis that is mediated by the protein, clathrin (Kirchhausen, Owen, and Harrison 2014). This molecule helps form the coated pit in the lumenal side of plasma membrane that buds to form a clathrin-coated vesicle. As a consequence of its actions, the vesicle includes not only the endocytosed material, but also extracellular fluid (Benmerah, Alexandre, and Christophe 2007; Rappoport 2008). On the other hand, endocytosis can also occur independent of clathrin, which can form caveolae or polymorphous tubes to engulf extracellular material (Hansen and Nichols 2009). Nonetheless, while the starting fates of these vesicles differ, their final fates all end at the lysosome.

Once the cargo is trapped within the endosome, the endosome moves to deliver the contents to the lysosome, along the endolysosomal pathway (Sorkin and von Zastrow 2009). Aspects of this pathway are incompletely defined and several remain controversial, with models proposing either a maturation of the endosome to the lysosome or a fusion between the endosome and the lysosome. In both models, one of the necessary steps for endolysosomal pathway is the acidification of the vesicular lumen, to a pH of approximately 5 (Ohkuma and Poole 1978). In comparison, a true lysosome will have a lumenal pH of 4.5-5 (Mindell 2012). The

pH drop is necessary for the delivery of acid hydrolases by mannose-6-phosphate receptor (M6PR) into the endosomal lumen (J. Paul Luzio et al. 2007). To achieve the right pH levels, v-ATPase, Clc7, mucolipin 1 (MCOLN1 or TRPML1), two pore calcium channel 1 (TPC1) and 2 (TPC2) pump protons, transport cation into and anions out of the maturing endolysosomal lumen (see previous section, "Lysosome structure"). Once all the hydrolases and the various membrane proteins are delivered to the developing endolysosome, the vesicle matures into the lysosome and loses expression of M6PR (J. Paul Luzio et al. 2007).

Intracellular cargo can be delivered to the lysosome through a process known as autophagy. Autophagy can be additionally categorized to three groups: microautophagy, chaperone-mediated autophagy (CMA), and most famously in mammalian cells, macroautophagy. Microautophagy, most commonly found in prokaryotic cells, describes the cellular phenomenon of direct consumption of proteins by the lysosome (Mijaljica, Prescott, and Devenish 2011). CMA is far more selective, where proteins need to be transported to the lysosome with the help of a chaperone. Here, the transported protein requires unfolding in order to get through the lysosomal membrane through the help of lysosome-associated membrane protein 2A (LAMP2A)(Sahu et al. 2011; A. M. Cuervo and Dice 1996). Mutations in this gene results in a lysosomal storage disorder known as Danon Disease, which disproportionately affects striated muscles, resulting in severe muscle weakness and early mortality (Rowland et al. 2016). Finally, macroautophagy, herein referred to as autophagy, uses an additional vesicle known as the autophagosome, a double membrane bound organelle that is capable of sequestering cytoplasmic material before fusing with lysosomes (He and Klionsky 2009; Noda and Inagaki 2015). In a way, autophagosomes resemble

that of the endosome, only designed for intracellular rather than extracellular cargo delivery.

Secretion

Lysosome-mediated exocytosis describes the specialized secretion of selective lysosomal contents into the extracellular environment. Although less studied than other functions of the lysosome, this mechanism is important in the bulk delivery of cargo and can be found in all cells (Rodríguez et al. 1997; Chavez, Miller, and Moore 1996; Coorssen, Schmitt, and Almers 1996; J. C. Stinchcombe and Griffiths 1999; N. W. Andrews 2000; Jaiswal, Andrews, and Simon 2002). Lysosomal exocytosis is particularly important in immune cell defense (Logan, Odemuyiwa, and Moqbel 2003; Wesolowski and Paumet 2011), bone remodeling (Mostov and Werb 1997), cell communication (Shin, Lee, and Jung 2012), pigmentation (J. Stinchcombe, Bossi, and Griffiths 2004), and plasma membrane repair (Castro-Gomes et al. 2016).

Calcium concentration is the central determinant of lysosomal secretory behavior. Increases in intracellular calcium triggers exocytosis of lysosomes. Proteins involved include VAMP7, a vesicle SNARE protein, SNAP23, a target SNARE, the calcium sensor synaptotagmin VII (SYTVII), syntaxin 4, and RAB proteins (Rao et al. 2004; Jahn and Scheller 2006; Bossi and Griffiths 2005). Additionally, the calcium channel MCOLN1 (or TRPML1, transient receptor potential cation channel) regulates at least a subset of lysosomal exocytosis events (LaPlante et al. 2006). Other potential regulators of this phenomenon may also include autophagy proteins: Microtubule-associated protein light chain 3 (LC3) is essential for lysosomal secretion by allowing the lysosome to fuse with the plasma membrane. Nonetheless, although LC3 is classically seen as an autophagy protein,

autophagy itself may not be involved in the direction of lysosomal secretion (DeSelm et al. 2011).

One of the key features of lysosome-mediated exocytosis is plasma membrane repair and remodeling. Injuries to the plasma membrane results in calcium entering cells through the injury lesions. Sudden and rapid increases in calcium provokes an almost immediate response by the lysosomes. The resultant secretory lysosomes carrying acid sphingomyelinase (ASM) fuse with the plasma membrane at the damaged sites, creating "patches" to reseal the lesions. This response by the cell happens within seconds and is necessary to prevent bacterial and protozoan pathogen invasion (Norma W. Andrews 2002; N. W. Andrews 2000; Reddy, Caler, and Andrews 2001; Huynh et al. 2004; Gerasimenko, Gerasimenko, and Petersen 2001).

Defects in lysosomal secretion through mutations in any of the various aforementioned players can result in impaired immune responses (Carmine Settembre, Carmine, Alessandro, et al. 2013), osteopetrosis (Lacombe et al. 2013), neurodegenerative diseases (Klein et al. 2005), and lipid storage disorders (Samie and Xu 2014).

Signaling

Adding to its repertoire of functions, the lysosome is also a regulator of cellular homeostasis. As the major site for cellular components and macromolecules, the lysosome is uniquely positioned as the cell's nutrient sensing center. Contents of the lysosome captures the cell's metabolic state at any given moment as the degradative products from the trafficked cargo are delivered to the organelle. When the a class of lysosomal substrates, i.e., proteins, is low within the cell,

then the degradative products, i.e., amino acids, are also low within the lysosomal lumen. Therefore, the lysosome is central to maintaining cellular homeostasis, particularly its metabolism and growth. A major protein complex on the lysosome is the **ly**sosome **nu**trient **s**ensing machinery (LYNUS), consisting primarily of mechanistic target of rapamycin complex 1 (mTORC1) (See Figure 1.3) (Carmine Settembre, Carmine, Alessandro, et al. 2013; Nnah, Khoosheh, and Radek 2015). As a master controller of cell growth and catabolism, mTORC1 is activated by anabolic factors such as growth factors, insulin, amino acids, oxygen, and glucose (Laplante and Sabatini 2012). Conversely, mTORC1 is inhibited by catabolic factors such as starvation, leading to the quelling of growth to save energy (Carmine Settembre, Carmine, Alessandro, et al. 2013; Nnah, Khoosheh, and Radek 2015; Efeyan, Zoncu, and Sabatini 2012; Mony, Benjamin, and O'Rourke 2016).

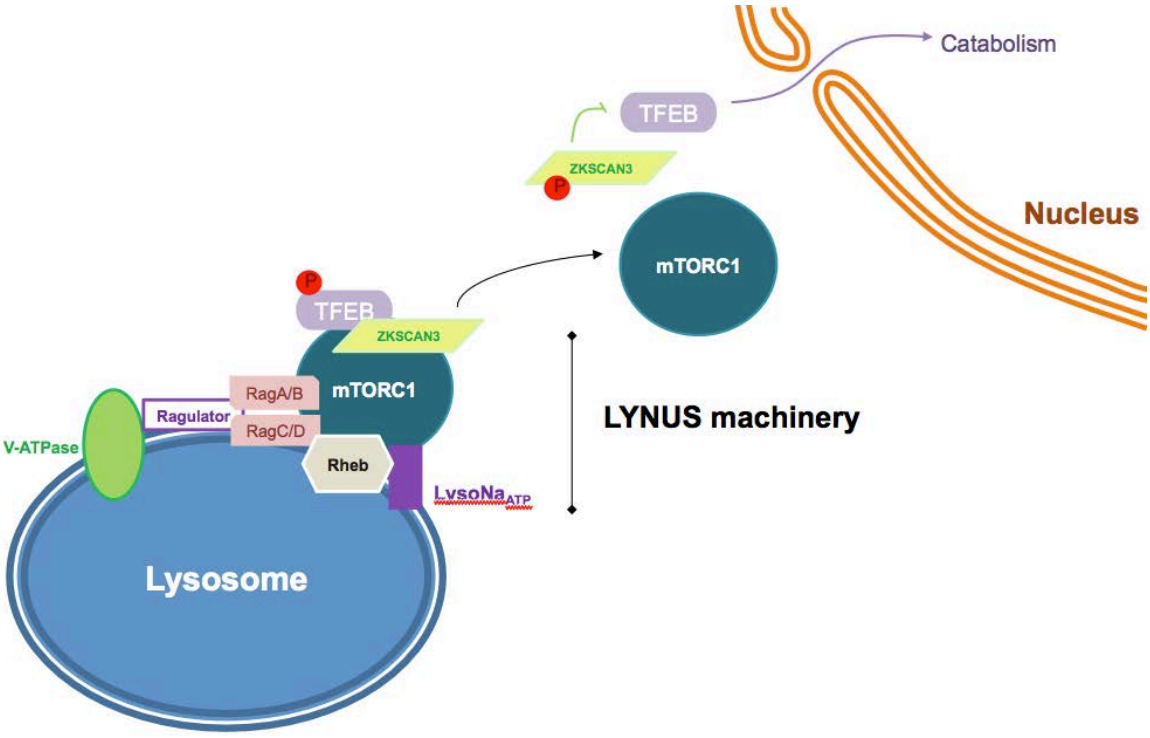

Figure 1.3: Lysosome nutrient sensing machinery

LYNUS permits the lysosome to play a key role in the metabolic homeostasis of cells. As cargo is shuttled to the lysosome, the lumen provides a measure of insight to the metabolic status of the cell. Increases in substrate levels would reflect the availability of such substrates for the cell. Indeed, mTORC1 and the lysosome are connected by at least theorized one amino acid sensor(Zoncu et al. 2011) that involves V-ATPase, and likely by glucose and lipid sensors as well. For mTORC1 to bind and come to the surface of the lysosome, amino acids must accumulate within its lumen. An accumulation of amino acids beyond a critical concentration activates mTORC1, thereby promoting the utilization of amino acids and anabolic pathways including mRNA translation, *de novo* lipid

synthesis, *de novo* synthesis and other pathways necessary for anabolic cell growth and proliferation.(Laplante and Sabatini 2012; Yu et al. 2010) As the degradative products from the lysosome are utilized by the cell, amino acid levels drop within the lysosome, eventually leading to the inactivation of mTORC1 . Discussion of mTORC1 signaling is further explored in the "Autophagy" section of this chapter.

The amino acid sensor has been proposed, and its existence only indirectly shown up until recently, when Rebsamen *et al.* found that the amino acid transporter, member 9 of the solute carrier family 38 (SLC38A9) interacted with various proteins involved in mTORC1 activation. Overexpression of SLC38A9 enabled mTORC1 activity to persist even under extreme starvation. SLC38A9 likely interacts with v-ATPase to enhance this phenomenon, although the exact mechanisms are still unclear. The endolysosomal ATP-sensitive Na^+-permeable channel, $lysoNa_{ATP}$, formed by two-pore channels (TPC1 and TPC2), may be an additional player connecting the lysosome with mTORC1. When mTORC1 is liberated from the lysosomal membrane during starvation, $lysoNa_{ATP}$ opens, regulating both amino acid homeostasis and lysosomal pH stability (Rebsamen et al. 2015; Zoncu et al. 2011). The existence of this channel allows lysosomes to respond to cytosolic ATP levels and couple them to nutrient availability (Zoncu, Efeyan, and Sabatini 2011).

Complexity of the nutrient sensing machinery and its dependence on the lysosome demonstrate the expansive nature the lysosome. Our understanding of lysosome structure and function are evolving rapidly, providing previously unrecognized roles of lysosomes in cellular homeostasis. This dissertation hopes to add another layer to the complex and functional repertoire of the lysosome.

Lysosomal regulation

Lysosomes require the full function of over a hundred proteins, not only for the structural and degradative maintenance of the organelle, but also for the regulation of its signaling operations. The large number of proteins therefore require coordinated and organized regulation. Microarray data have recently identified a "lysosomal gene network" that coordinates the production of proteins required for lysosome assembly. This gene network along with the regulators of lysosome have provided insights to how the cell manages to govern one of the most intricate and complex organelles it houses.

CLEAR Network

The identification of the lysosomal gene network, also known as the coordinated lysosomal expression and regulation (CLEAR) network, demonstrates the global control and coordination of the transcription of lysosomal associated genes. A systems biology approach to microarray data of known lysosomal genes uncovered a 10-base, palindromic sequence, within the promoters of many lysosomal genes. This site, an E-box, allows for the binding of transcription factors belonging to the basic helix-loop-helix (bHLH) (Palmieri et al. 2011). Discovery of the CLEAR network is a major tool that allows researchers to expand their knowledge of lysosomal regulation (Carmine Settembre and Medina 2015).

TFEB

Transcription factor EB, or TFEB, was only recently recognized as a master regulator of lysosomal function and biogenesis (Medina et al. 2011). As a global regulator of lysosomes, TFEB exerts transcriptional control over lysosomal and autophagosome biogenesis, lysosome degradation and exocytosis, substrate

targeting, and fatty acid oxidation (C. Settembre et al. 2011; Carmine Settembre, Carmine, De Cegli, et al. 2013; Carmine Settembre, Carmine, and Andrea 2011; C. Settembre et al., n.d.; Carmine Settembre et al. 2012; Roczniak-Ferguson et al. 2012).

Under normal fed conditions, mTORC1 sits on the lysosomal membrane and phosphorylates TFEB at conserved serine residues. This action by mTORC1 sequesters TFEB to the lysosomal surface, preventing its translocation to the nucleus (Sardiello et al. 2009; C. Settembre et al. 2011). While there are at least ten phosphorylation sites identified on TFEB, only two, Ser211 and Ser142, have been shown to be necessary for the sequestration of TFEB in murine models (Carmine Settembre et al. 2012; Jose A. Martina et al. 2012; Roczniak-Ferguson et al. 2012). Conversely, under fasting conditions, the nutrient status signalled by the lysosome inactivates mTORC1 via Ragulator, thereby inhibiting mTORC1's actions on TFEB. Non-phosphorylated TFEB, now free of mTORC1's localization control, moves to the nucleus binding promoters of genes that modulate various aspects of lysosome regulation, autophagy, and lipid catabolism (See Figure 1.4) (Maxfield, Willard, and Lu 2016).

TFEB is a member of the microphthalmia-associated transcription factor (MITF) group of transcription factors, a subfamily of bHLH transcription factor family (Massari and Murre 2000). TFEB binds to the CLEAR sites of lysosomal associated genes, located in the promoter region (Carmine Settembre and Medina 2015). Interestingly, based on data from *C. elegans* studies, the TFEB orthologous gene, *hlh30*, acts in complement to MAX-like protein X 3 (MLX-3) . A transcriptional repressor, MLX-3 sits on the promoter region of lysosomal associated genes, in particular, the *C. elegans* lysosomal lipases, *lipl1* and *lipl3*.

Fasting degrades MLX-3 protein levels, allowing for HLH30 to bind to the CLEAR sequence and transcribe genes in the CLEAR network (Maxfield, Willard, and Lu 2016; Carmine Settembre, Carmine, De Cegli, et al. 2013; O'Rourke and Ruvkun 2013; Lapierre et al. 2011).

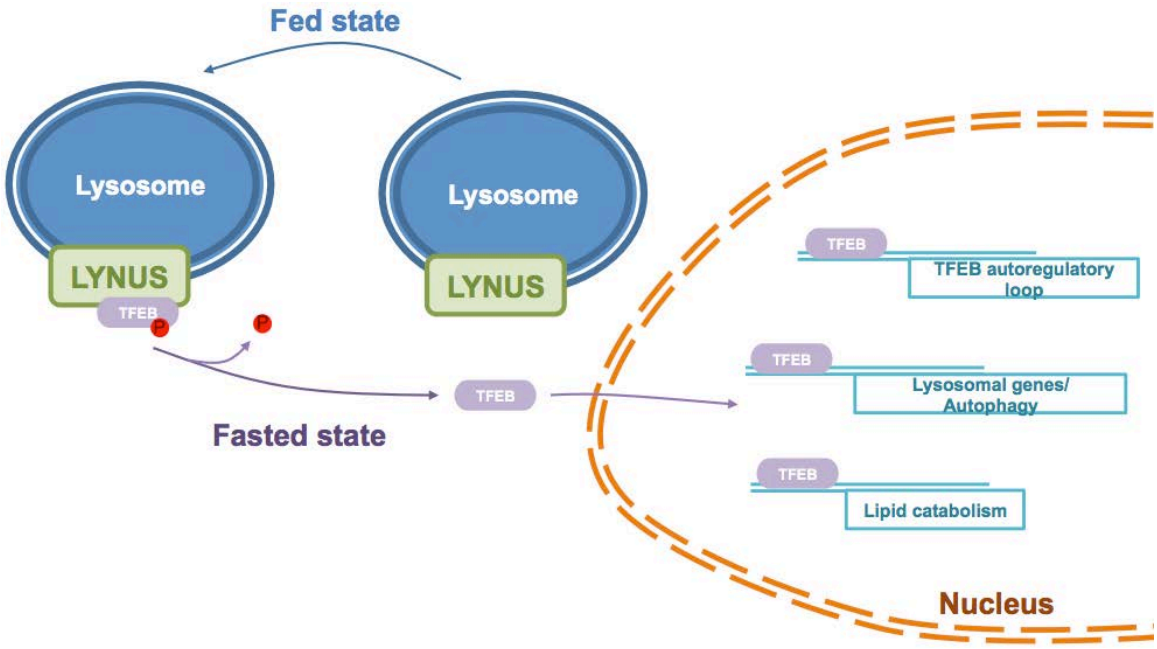

Figure 1.4: The lysosome communicates with the nucleus via the actions of TFEB.

MITF/TFE3

In addition to TFEB, MITF/TFE3 are two members of the bHLH transcription factor family that have been implicated in the regulation of lysosomal biogenesis and function (Massari and Murre 2000; Meadows et al. 2007; Rehli et al. 1999; Mansky et al. 2002; José A. Martina et al. 2014; Steingrímsson et al. 2002). Their

relationship to mTORC1 and anabolism is evidenced by their association with melanomas (Feige et al., n.d.; Arnheiter and Heinz 2010), renal carcinomas (LaGrange, Lele, and Strup 2007; "Renal Cell Carcinoma Associated with Xp11.2 Translocation and TFE3 Gene Fusions" 2012; Fall et al. 2011), and alveolar soft part sarcoma (Hodge et al. 2013). These two bHLH transcription factors also share extensive sequence homology with TFEB, further evidence for their roles in lysosomal regulation. In fact, both MITF and TFE3 interact with TFEB via dimerization and participate in subcellular translocation to activate downstream lysosomal associated genes (Jose A. Martina and Puertollano 2013; José A. Martina et al. 2014; Steingrímsson et al. 2002). This close interaction, however, implies that analyzing the separate and independent functions of each transcription factor is difficult (Jose A. Martina and Puertollano 2013; José A. Martina et al. 2014).

While mTORC1 phosphorylates TFEB, other proteins involved in cellular housekeeping seem to regulate the actions of MITF. Both mitogen-activated protein kinase (MAPK) and wingless-related integration site (WNT) proteins phosphorylate and inhibit MITF. Although the same conserved sites have been found on the sister proteins, TFEB and TFE3, it is unclear whether or not they also interact with MAPK and WNT (Ploper and De Robertis 2015).

FOXOs

The forkhead box O (FOXO) transcription factor family has also been implicated in the regulation of lysosome function through its effects on autophagy via mTORC1 inactivation (Demontis and Perrimon 2010; Calnan and Brunet 2008; Zhao et al. 2007; C.-C. Chen et al. 2010). A catabolic protein that serves as a foil for insulin action, FOXOs are active during nutrient deprived states, similar to

TFEB/MITF/TFE3. Binding of the insulin receptor by circulating insulin activates the phosphoinositide 3-kinase - protein kinase B (PI3K-AKT) pathway. All proteins in the FOXO family contain multiple AKT phosphorylation sites; phosphorylation of these sites through the induction of 14-3-3 binding (Obsil et al. 2003; Silhan et al. 2009) prevents DNA binding, thereby inhibiting the transcriptional ability of FOXOs. Conversely, inhibition of downstream insulin signaling cascades (PI3K/AKT and MAPK/ERK) activates FOXOs (Roy, Srivastava, and Shankar 2010). Hence, anabolic signals directly prevent the actions of FOXOs.

ZKSCAN3

Similar to MITF and TFE3, zinc-finger protein with KRAB and SCAN domains 3 (ZKSCAN3) initially came to the attention of researchers as a result of its role in cancer pathogenesis, specifically colorectal cancer (Lin Yang et al. 2008). Later studies demonstrated a role for ZKSCAN3 in multiple myeloma (L. Yang et al. 2011), plasma cell leukemia (J. B. Egan et al. 2012), and prostate cancer (X. Zhang et al. 2012). Finally, five years after its initial incrimination as an oncogene, ZKSCAN3 was found to be a master repressor of autophagy and lysosome function, antithetical to TFEB (Chauhan et al. 2013). Among the genes affected by ZKSCAN3 include *Lc3* and *Wipi12* (WD repeat domain, phosphoinositide-interacting 2). Loss-of-function experiments show that ZKSCAN3 knockdown by itself can increase autophagy and lysosomal biogenesis; gain-of-function likewise demonstrated the opposite effect. Therefore, ZKSCAN3 and TFEB serve complementary roles in the regulation of cellular anabolism and catabolism (Chauhan et al. 2013).

Autophagy

MTOR is an important regulator in many pathways, but particularly those involved in metabolism and cell growth. Insulin, the central peptide hormone in regulating carbohydrate and lipid metabolism in the body, can activate mTOR via both the PI3K/AKT and MAPK/ERK pathways. Insulin binds to its receptor tyrosine kinase, insulin receptor (IR), which phosphorylates insulin receptor substrate 1 (IRS-1). Phosphorylation of IRS-1 eventually leads to the activation of AKT. Under normal circumstances, the tuberous sclerosis proteins 1 and 2 (TSC1-TSC2) form a complex that inhibits the GTPase activity of the G-protein, ras homolog enriched in brain (RHEB), by hydrolyzing RHEB-GTP into its inactive RHEB-GDP bound off state. However, AKT phosphorylates TSC1-TSC2, preventing the complex from exerting its inhibitory functions on RHEB. Consequently, RHEB stays in its GTP-bound on state and activates mTOR, resulting in the activation of a number of cellular pathways, including autophagy (Zoncu, Efeyan, and Sabatini 2011; Laplante and Sabatini 2012).

Autophagy is necessary for cell survival during nutrient deprivation. As a result, not surprisingly, mTOR regulate autophagy. In autophagy, cellular components are broken down by first sequestering them into vesicles known as autophagosomes. These double lipid bilayer membrane vesicles are likely derived from the endoplasmic reticulum, Golgi apparatus, mitochondria, and plasma membrane (Singh and Cuervo 2011; Ana Maria Cuervo 2010). These autophagosomes then fuse with lysosomes, forming autophagolysosomes. This newly formed vesicle will contain the same enzymes found in the lysosome that can degrade the proteins and release its products into the cytoplasm for reuse (Singh and Cuervo 2011).

One of the key regulators of autophagy, unc-51-like kinase 1, ULK1, regulates membrane nucleation, allowing the cellular components to become sequestered into autophagosome. Under nutrient depletion, 5' adeonsine monophosphate-activated protein kinase (AMPK) phosphorylates ULK1 on at least four sites, three of which have been identified as serine 555, threonine 574, and serine 637 (D. F. Egan et al. 2011). Phosphorylation of these sites activate ULK1 activity by an unknown mechanism, allowing it to form a complex with its subunits, autophagy-related protein 13 (ATG13) and focal adhesion kinase family interacting protein of 200 kD (FIP200). Moreover, ULK1 governs autophagy-related protein 101 (ATG101) and Beclin1/class III phosphatidylinositide 3-kinase (PI3K) VPS34, necessary for the generation of the autophagosome membrane. mTOR suppresses ULK1 activity via phosphorylation of the protein at serine 757 (Kim et al. 2011). Without ULK1 function, autophagy is inhibited. Unlike other degradative processes such as apoptosis, autophagy is primarily a protective system that breaks down both damaged cytoplasmic products as well as undedicated nutrient stores to meet the energy demands of the cell during resource deprivation (Madeo, Tavernarakis, and Kroemer 2010).

Other key autophagic proteins include SNAREs, vacuolar protein sorting-associated protein (VPSs), and RABs. These proteins' peak functions occur during the biogenesis of autophagosomes and are associated with mTORC1 activity as well (Morelli et al. 2014; Moreau, Renna, and Rubinsztein 2013; Ao, Zou, and Wu 2014; Bento et al. 2013; Popovic et al. 2012). Many of these genes involved in autophagosome biogenesis are additionally regulated by TFEB and FOXOs (Carmine Settembre, Carmine, Alessandro, et al. 2013).

As a result of its cytoprotective functions, autophagy has been well implicated in both aberrant metabolism and accelerated aging (Carmine Settembre, Carmine, Alessandro, et al. 2013; Rubinsztein, Guillermo, and Guido 2011). Insulin resistance in humans, for example, correlates with a decreased efficacy in the autophagic machinery (de Kreutzenberg et al. 2010). Hepatic lipid metabolism is in part mediated by autophagy, where it breaks down neutral lipids via lysosomal acid lipase (LIPA) (Singh, Kaushik, et al. 2009). Obesity disrupts this process, ultimately promoting hepatic insulin resistance (Singh, Kaushik, et al. 2009). In the pancreas, the β-cells require intact autophagy for their expansion during high fat diet and insulin resistant states (Quan, Lim, and Lee 2012; Linnemann, Baan, and Davis 2014; Ebato et al. 2008).

Lipid catabolism and Lipophagy

Early studies of autophagy and lysosomes focused on degradation of protein and complex carbohydrates. However, in the last decade, researchers have identified critical roles for lysosomes in lipid catabolism (Singh, Kaushik, et al. 2009; Carmine Settembre, Carmine, De Cegli, et al. 2013).

The significance of lysosomes in lipid metabolism is particularly obvious in aged and obese mice (Cota and Daniela 2009). Increases in age and adiposity hyperactivate mTORC1, making it less sensitive to nutrient signals provided by the lysosome (S.-B. Yang et al. 2012). Lipidomic studies show that these mice accumulate lipids in their lysosome (Rodriguez-Navarro et al. 2012). Furthermore, their lysosomes act as if partially disabled, with altered hydrolysis of components, cholesterol accumulation, and inability to partake in fusion as

well as fission. These abnormalities further exacerbate altered mTORC1 actions and its subsequent inappropriate downstream signaling (Rodriguez-Navarro et al. 2012; Carmine Settembre and Ballabio 2014).

Lipophagy

Briefly, lipophagy is a form of autophagy that allows the cell to degrade lipid droplets (LDs). As a selective form of autophagy, lipophagy describes a vesicular alternative to cytosolic lipolysis by hormone sensitive lipase (HSL) and adipose triglyceride lipase (ATGL). The lipid droplet of the adipocyte, contrary to earlier beliefs, is a dynamic organelle covered by various structural and regulatory proteins that allow it to not only respond to cellular environmental changes, but also interact with other organelles (Greenberg et al. 2011; Fujimoto and Parton 2011; Murphy et al. 2009). However, because the lipid droplet contains a protein coat and not a lipid bilayer, it cannot directly fuse with the lysosome for its contents to be hydrolyzed by lysosomal acid lipase (LIPA). Therefore, lipid droplet sequestration can only be performed by the autophagosome, which contains the necessary machinery to take in such cellular components. Key players in autophagy, in particular autophagy proteins 5 and 7 (ATG5 and ATG7), localize to fractions of lipid droplets, aiding in the formation of a limiting membrane at the lipid droplet. With the help of autophagy protein 12 (ATG12) and LC3 conjugation to phosphatidylethanolamine (PE), phospholipids begin to create the limiting membrane layer from the surface of the lipid droplet. Once the autophagosome carrying the lipid droplet fraction is fully mature, it can then fuse with a lysosome to deliver the lipid based contents (Singh, Rajat, and Cuervo 2012).

Previously, lipid breakdown in the cell was thought to occur exclusively in the cytosol by HSL, ATGL and monoacylglycerol lipase (MGL). However, studies in

autophagy debunked this limited view. Cells with dysfunctional autophagy toxically accumulate LDs in the cytoplasm. Patients suffering from hepatic steatosis exhibit not only excess LDs within hepatocytes, but also the presence of lipid filled lysosomes. Further, LIPA deficiency, a spectrum of disease from severe (complete lack of activity - Wolman disease) to moderate (partial lack of LIPA activity - Cholesteryl ester storage disease, CESD) (Aslanidis et al. 1996). Wolman disease patients often die before reaching one year of age from impaired intestinal function, liver failure, and immune defects. In contrast, CESD patients suffer from varying degrees of hyperlipidemia, premature cardiovascular disease, and hepatic steatosis that can progress to liver failure (B. Zhang, Bingnan, and Porto 2013; Barness 1996, 2563–2587). The presence of neutral lipases in these cases fail to defend the cell and organism; lysosomal-dependent lipolysis, including lipophagy, therefore is required for lipid homeostasis.

Further connecting lysosomes to lipid degradation, the master lysosomal regulator TFEB, is a transcription factor for a number of lipid metabolism genes. These genes code from proteins that include: cluster of differentiation 36 (CD36), fatty acid binding protein (FABP) (for FA import through the plasma membrane), carnitine palmitoyltransferase 1 (CPT1), carnitine acetyltransferase (CRAT), acyl-CoA dehydrogenases (ACADL and ACADs), (for mitochondrial b-oxidation of FAs), cytochrome p450, family a, subfamily 4 (CYP4a), LIPA, and more importantly, PGC1α and PPARs (master regulators of lipid catabolism) (Carmine Settembre, Carmine, Alessandro, et al. 2013). Overexpression of TFEB in the liver alleviated symptoms of metabolic syndrome (Carmine Settembre, Carmine, Alessandro, et al. 2013). Similar gain-of-function mutations in TFEB in macrophages delayed atherogenesis (Emanuel et al. 2014). Therefore, in addition

to controlling lipophagy, TFEB also ties other themes in lipid degradation to the lysosome.

Lysosomal contribution to lipid catabolism is at its peak during nutrient deprived states, at least in the liver. Its contribution to meeting the energy of other cell types, particularly adipocytes, remain uncertain and controversial. For example, lysosomes in adipose tissue were originally assumed to not be of any unique or specialized importance. Yet, adipose tissue specific knockout of ATG7 results in reduced adipocyte lipid droplet size, tissue mass, and impaired adipogenesis (Singh, Xiang, et al. 2009). Moreover, these mice also exhibit brown adipose tissue like features in their white adipose tissue. However, due to inherent limitations in the genetic model, it is unclear if these features are results of developmental problems; a direct role for lipophagy in adipocyte lipid catabolism is not clear. In fact, data from our lab demonstrate that preadipocytes isolated from LIPA deficient and wildtype littermates differentiate similarly *in vitro*, contradicting the specific roles of lipophagy in adipocyte differentiation.

Febrile Response

A fever is a classic response to inflammation and many infections. Research on the study of the febrile response has revealed many complex heat sensitive mechanisms that assist the immune system during defense. Nonetheless, despite our knowledge regarding these immune processes, the source of heat during a fever is still an incompletely solved mystery.

The induction of fever from infection is currently thought to be dependent on the hypothalamic response to interleukin 6 (IL-6), which function downstream of the other major pyrogenic cytokine, IL-1 (See Figure 1.5) (Dinarello et al. 1991;

Kozak et al. 1998; Netea, Kullberg, and Van der Meer 2000). Stimulation of the medial preoptic nucleus of the hypothalamus by IL-6 results in additional prostaglandin E2 (PGE2) (Matsumura et al. 1998; Yamagata et al. 2001; Engström et al. 2012). Following stimulation of the PGE receptor 3 (EP3) expressing neurons by PGE2, the sympathetic nervous system triggers the release of norepinephrine, activating two responses that work together to increase body temperature by (1) reducing heat exchange and (2) increasing heat generation (Engblom et al. 2003; Lazarus et al. 2007). The reduction in heat exchange in mammals involves decreasing circulation to the skin and reducing evaporation based heat exchange. There is also strong evidence for increased heat production through both shivering and nonshivering thermogenic mechanisms (Evans, Repasky, and Fisher 2015) (Figure 1.5).

While many models of febrile response include activation of BAT, it is unclear what contribution BAT has on raising body temperature, especially in adults. A study from the Saito Lab provided evidence that in the absence of UCP1, mice are still able to mount a febrile response, putting into question the necessity of BAT function in febrile response (Okamatsu-Ogura et al. 2007). Furthermore, given the low BAT mass in humans, it seems likely that UCP1 independent mechanisms are involved during fever induction.

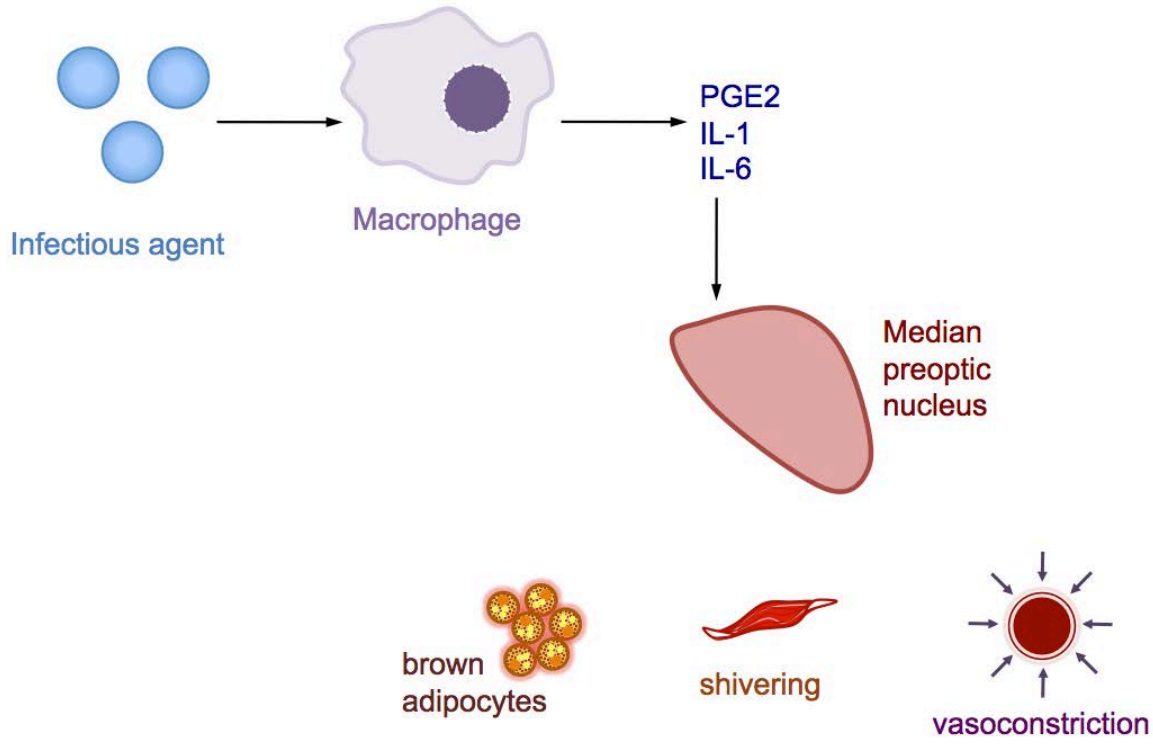

Figure 1.5: Brief overview of the febrile model

Hypothesis

In studies of lysosomal acid lipase, we discovered that lysosomes are critical for thermogenesis and propose that they contribute to the regulation of heat generation in three ways: 1) As regulatory organelles that modulate transcription necessary for UCP1-dependent thermogenesis; 2) By providing substrates for uncoupled oxidation; 3) As thermogenic organelles that generate heat through catabolism of macromolecules.

Proposed Models

Model #1: Lysosomes indirectly regulate lipid catabolism and NST genes through the actions of lysosome regulators such as TFEB/TFE3/MITF transcription factor family.

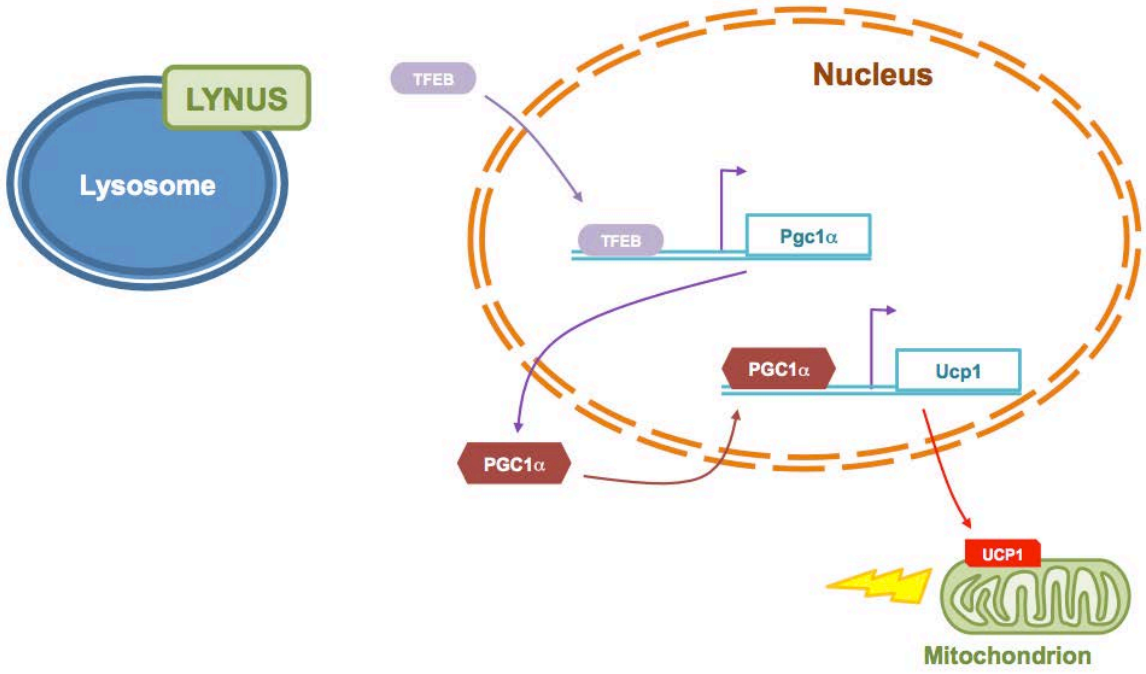

Figure 1.6: Lysosomes are signaling regulators of nonshivering thermogenesis

Rationale:

As a recently discovered major signaling center of the cell, the lysosome is home to a number of critical metabolic proteins. TFEB, in addition to other members of the CLEAR domain binding transcription factor family, is intimately associated with lysosomal activity via mTOR. TFEB is upstream of a number of pathways, including lipid catabolism, via the actions of PGC1α. As a crucial

transcription factor for UCP1, PGC1α can subsequently regulate NST. Therefore, we hypothesize that lysosomes contribute to NST activation via TFEB and other members of the CLEAR transcription factor family.

Description of signaling model:

Cold exposure demands profound levels of energy from the brown adipocyte as the efficiency of ATP production drops to compensate for the production of heat. As a result, food intake and catabolism must increase to support the sudden rise in energy demand. This changed state alerts the LYNUS machinery to activate TFEB to increase transcription of catabolic genes, including PGC1α. Consequently, UCP1 transcripts rise, resulting in NST activation.

Model #2: Lysosomes contribute to heat generation directly as thermogenic organelles.

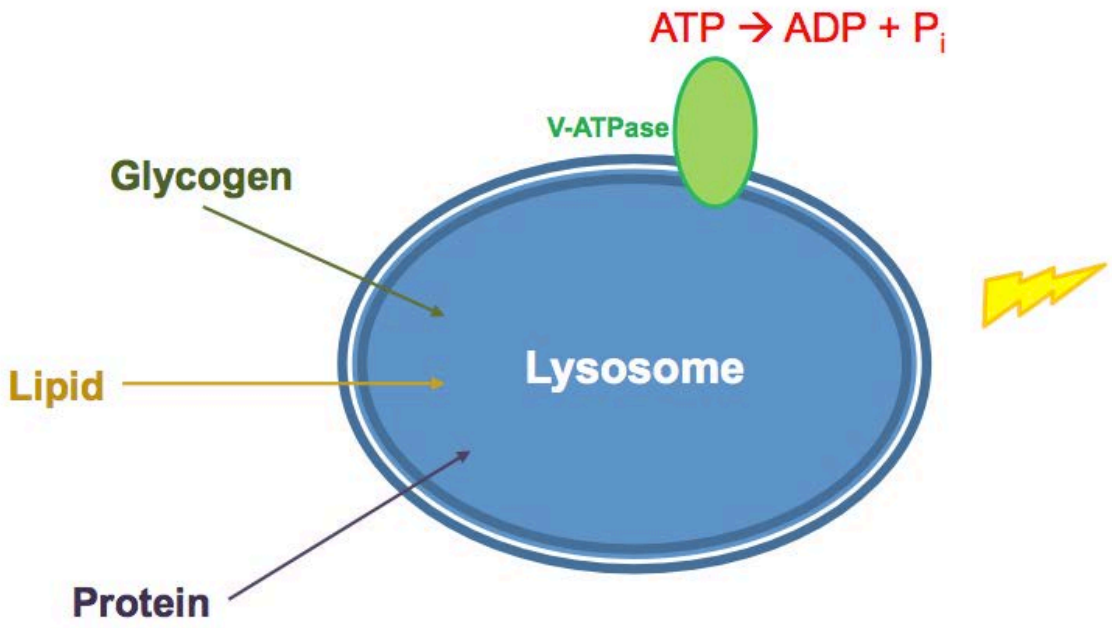

Figure 1.7: Lysosomes are thermogenic organelles.

Rationale:

Thermodynamics deem that perfectly efficient reactions in nature do not exist; that is, entropy is always positive. While the laws of thermodynamics and entropy are generally constrained in the to the fields of physics and chemistry, they form the critical basis for thermogenesis studies. Nonshivering thermogenesis, as we currently understand it, is defined by the uncoupling of biochemical reactions from the creation of useful chemical energy such as ATP, resulting in heat dissipation. While there are two well known systems that participate in this phenomenon: UCP1 activation in brown adipocyte mitochondria as well as SERCA actions in stimulated muscle fibers, it is not known if other organelles such as lysosomes exhibit such physiology.

Further, many catabolic reactions like those that occur in the lysosome are exothermic, releasing small amounts of heat to abide by the second law of thermodynamics. By regulating delivery and degradation of high-energy macromolecules, cells can generate heat in a UCP1 independent fashion. Consequently, we hypothesize that any cell with lysosomes can generate heat. The question is not whether lysosomes are at all thermogenic, but rather are physiologically relevant thermogenic organelles.

Description of heat generation model:
Cold challenge induces substantial catabolism, increasing exothermic heat release to physiologically relevant levels.

Lysosomes provide substrates necessary for thermogenesis.

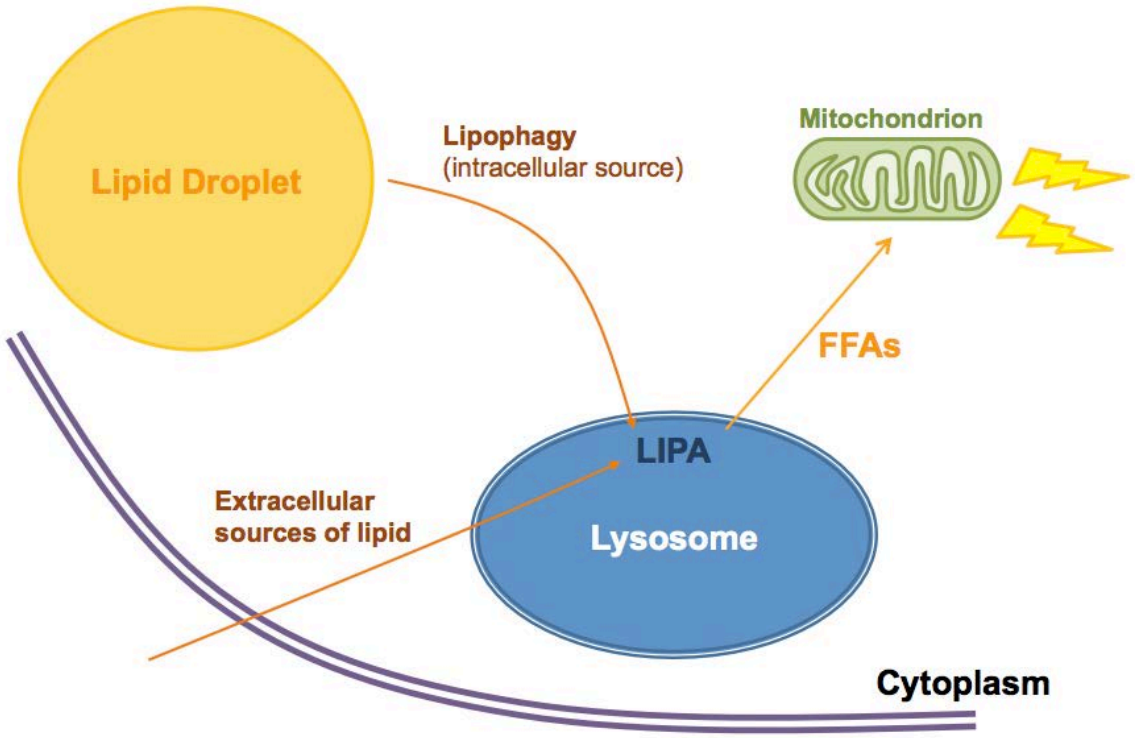

Figure 1.8: Lysosomes provide free fatty acid substrates necessary nonshivering thermogenesis

Rationale:

Generation of fatty acids for uncoupled oxidation is necessary for BAT thermogenesis. We hypothesize that lysosomes, through the action of lysosomal acid lipase (LIPA), hydrolyze triglycerides into free fatty acids and glycerol. Lysosomal derived free fatty acids serve as critical substrates for the mitochondrion during nonshivering thermogenesis.

Description of substrate model:

During cold challenge, lysosomes shuttle large amounts of free fatty acids to the mitochondria for NST use. Lipids may enter through classical means: lipophagy, reësterified triglycerides from the circulation, lipoproteins, CD3 uptake of lipids. These sources of lipids, once delivered to the lysosome, can then hydrolyzed by the residing lysosomal acid lipase. Subsequently, the free fatty acid products and glycerol can then be secreted out of the lysosome, and delivered straight to the mitochondria.

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CHAPTER TWO

LYSOSOMES ARE ASSOCIATED WITH BODY TEMPERATURE REGULATION

Introduction

Lysosomes are critically important for the regulation of cellular homeostasis (Settembre et al. 2013). Body temperature management likewise is equally necessary for the maintenance of whole body physiology. We hypothesized that lysosomes contribute to regulated thermogenesis in three ways: as signaling nodes that monitor substrate availability, as substrate providers for uncoupled oxidation and as heat generating organelles. If any aspect of our hypothesis is correct, then we predict that activation of nonshivering thermogenesis will activate lysosome function in thermogenic organs.

Organisms activate thermogenesis in response to drops in ambient temperature and as a physiological part of inflammatory responses. Homeotherms activate thermogenesis when the ambient temperature drops below the point at which basal metabolism and circulatory responses are insufficient to maintain body temperature - defined as the "thermoneutral temperature." There has been great interest in cold induced thermogenesis, and many studies largely center around UCP1-dependent uncoupled oxidation. In comparison, there have been relatively few studies of thermogenesis in febrile responses or in local heat generation during injury and inflammation. There have been even fewer studies of UCP1-independent thermogenesis, yet the existence of non-canonical thermogenesis is compelling. UCP1-deficient mice are able to mount a thermogenic response and survive in cold environments when previously acclimated (Nedergaard et al. 2001;

Golozoubova 2001). Similarly, birds lack functional UCP1 homologue, yet they live in some of the coldest environments on Earth (Emre et al. 2007). Additionally, animals with defective brown adipose tissue (BAT) thermogenesis, including ones with defective leptin signaling, are able to mount febrile responses that are indistinguishable from mice with intact BAT (Okamatsu-Ogura et al. 2007).

Regulation of body temperature, of course, is not limited to the traditional narrow scope and view of nonshivering thermogenesis. The raising of body temperature during a febrile response represents another facet to body temperature control. While the current febrile response model involves the actors PGE, IL6, and UCP1 (Evans, Repasky, and Fisher 2015), we hypothesized that lysosomes may be an additional influencer in the model. In other words, lysosomes may be important not only for the defense of body temperature, but also for its increase. Consequently, in this chapter, we also explore whether induction of a febrile response through infection mimicry could result in a likewise increase in lysosomal associated genes.

Finally, given the importance of whole body temperature homeostasis in nearly all animals, lysosomes may be a conserved undiscovered temperature regulating mechanism even in poikilotherms. While homeotherms have well established molecular defenses against a cold challenge, most adaptations by poikilotherms seem to be behavioral (Heath 1970). We propose, given the lack of Ucp1 expression in poikilotherms, that lysosomal activity may be a conserved evolutionary response to cold challenge.

Naturally, a mere association between the two phenomena does not describe a physiologically relevant and causative relationship. To establish such a rapport

between thermogenesis and lysosomes, we will explore whether disruption of lysosomes alters the response by thermogenic tissues. Both pharmacological and genetic approaches can be used to create a lysosomal dysfunctional phenotype. If these lesions are capable of altering the thermogenic actions of brown and beige adipocytes after a cold challenge, then lysosomes have either direct or indirect effects on cold sensitivity. Febrile responses in lysosomal disruption models will further corroborate the causal relationship between lysosomes and body temperature regulation.

Proposed Hypothesis and Model:

The lysosome has a correlative relationship between its activity and body temperature regulation.

Methods and Materials

Animals and Animal Care.

FVB/NJ mice were obtained from the Jackson Laboratory at 6-7 weeks of age, or from the laboratory stock of lysosomal acid lipase (LIPA) mice, originally generously provided by Hong Du, PhD of Indiana University. LIPA knockout mice are missing exon 5 of the LIPA gene, resulting in a dysfunctional LIPA transcript. Experiments were performed on littermates. Mice were housed in ventilated Plexiglas cages within specific pathogen-free barrier facility maintained in a 12-hour light/12-hour dark cycle. Regular water and chow diet were provided ad libitum. LIPA deficient mice were genotyped using polymerase chain reaction screening of tail DNA. All procedures were approved by the Columbia University International Animal Care and Use Committee.

Quantitative Real-time Polymerase Chain Reaction.

Tissue samples were immediately frozen by liquid nitrogen from euthanized mice at the termination of experiments. RNA was extracted from frozen tissue samples using an acid-phenol reagent according to manufacturer's instructions (RNA-solv reagent, Omega Bio-tek). RNA was further purified by silica-membrane method from the manufacturer (E.Z.N.A. Total RNA Kit II, Omega Bio-tek) and then used as template for cDNA synthesis (qScript cDNA SuperMix, Quanta Biosciences). Quantitative real time PCR (qRT), was performed using white 384 well plates in the Roche LightCycler 480 machine. Analysis was done by normalizing expression to Rps3 and expressed in relative units using the DDCT-2 method, taking account of each primer efficiency calculated per in-run standard curve. Primer sequences are listed in Table 1.

Immunoblots.

Tissue samples were homogenized by sterile stainless steel beads with QIAGEN's TissueLyser LT in tissue lysis buffer (Tissue extraction reagent I, Thermo Fisher) treated with protease and phosphatase inhibitors (Protease inhibitor cocktail I, phosphatase inhibitor cocktail II and III, SIgma Aldrich). Protein concentrations were determined by standard BCA assay (Bio-Rad). Crude protein extracts were centrifuged to separate the lipid fraction from the aqueous layer three times to purify protein. Purified protein extracts were gel electrophoresed using 10% SDS/PAGE gels, transferred to PVDF .2 um membranes (Millipore info), and immunostained according to standard methods. Antibodies used: UCP1 (info), LAMP1 (info), LAMP2 (info), PGC1 (info), PPARy (info), phospho-p38MAPK (info), p38MAPK (info), b-actin (info), a-tubulin (info). Blots were visualized using LI-COR fluorescent secondary antibodies and a Li-COR imager.

Protein densitometry.

Protein densitometry was calculated using ImageJ. ALl protein are normalized to either beta-actin or alpha-tubulin for whole cell or cytoplasmic fractions. TATA-binding protein (TBP) was used as the control protein for nuclear fractions.

Lysosomal acid lipase activity assay.

LIPA activity was determined using a 4-methylumbelliferyl oleate assay with 4-methyumbelliferone serving as the standard curve. 4-MUO was dissolved in hexane at 100 mg/ml and diluted 1 to 250 in 4% Triton X-100. Tissue extracts were prepared in the same way as western blot samples (see Immunoblots). 25 ul of diluted tissue extracts, 50 ul of diluted 4-MUO substrate, and 125 ul of assay buffer (0.2 M NaOAc and 0.01% Tween 80, pH 5) were incubated at 37flC for 30 minutes. To stop the reaction, 100 ul of 0.75 M Tris, pH 8.0 was added to each sample. Fluorescence was detected using at excitation 360 nm and emission 460

nm. Relative fluorescence units from each sample were then compared to that of the standard curve and normalized to wildtype controls.

Histology, Immunohistochemistry, and Immunofluorescence.

Histology was performed by Columbia University Medical Center Russ Berrie's Pathology Core. Sections were sliced at 5um and paraffin-embedded. For IHC staining, adipose tissues were fixed with Z-FIX for 48 hours and embedded in paraffin. Tissue sections (5mm thick) were deparaffinized, rehydrated, and followed by antigen retrieval step before being stained with ABC/DAB (Vectastain) kit followed by counterstain. Antibodies used for IHCs: Ucp1 (info). For IF staining, adipose tissues were fixed in 4% PFA for 24 hours, followed by 10% sucrose and embedded in OCT before freezing. Tissue sections (5mm thick) followed the standard IF protocol described by AbCam. IF slides were mounted using Invitrogen's ProLong Gold antifade reagent with DAPI . Antibodies used: LAMP1 (eBioscience), LAMP2 (Abcam), PLIN1 (Abcam), and M6PR (Abcam).

Brown adipose tissue isolation.

Brown adipocytes were isolated from extracted whole interscapular brown adipose tissue. Tissue were minced such that individual pieces were ¡1 mm or less in diameter in 350 ul of cell medium, resulting in a tissue slurry. Following mincing, the tissue slurry was placed in cell medium with liberase (Roche) and incubated at 37C with shaking for 30-50 minutes, depending on the efficiency of the liberase stock. The resulting solution was passed through a 100 micron cell strainer (Gibson) and centrifuged at 500g for 10 minutes. The supernatant was removed and the cells were reconstituted in 5 mLs of cell medium before being centrifuged again at 500 g for 10 minutes. Cells were then reconstituted in cell medium and counted for cell concentration.

Splenocyte isolation.

Splenocytes were isolated from whole spleens extracted from sacrificed mice. Following extraction, splenocytes were placed in a plate containing a 70 micron cell strainer immersed in cell medium. A 10 mL syringe plunger was used to press the whole spleen within the cell strainer against the plate wall. After filtering the ground tissue through the cell strainer, cells were centrifuged at 500g for 5 minutes. Following centrifugation, cells incubated in a lysis buffer for five minutes at room temperature (Invitrogen) to lyse the erythrocytes. The suspension was then pelleted again via centrifugation at 500g for an additional 5 minutes before resuspending again in two mLs of cell medium. Cells were counted for concentration prior to ITC measurements.

Chloroquine injections.

Chloroquine (Sigma-Aldrich), a lysosome inhibitor, was injected either intraperitoneally or directly into the BAT tissue at 3.5 mg/kg.

CL 316,243 injections.

CL-316,243 (Sigma-Aldrich), a beta3-adrenergic receptor agonist, was injected intraperitoneally at 1 mg/kg. Mice were sacrificed 2 hours post injection.

Liver isolation.

Portions of the right anterior and left medial segments of the murine liver was removed from the whole liver for each isolation, unless otherwise specified.

Skeletal muscle isolation.

Whole soleus and extensor digitorum longus muscles were removed from each hindlimb of a mouse our studies.

Lipopolysaccharide injections.

Lipopolysaccharide (Sigma-Aldrich L5418-2ML) was injected intraperitoneally at 10 ug/kg and monitored every two hours (unless otherwise specified) via a temperature probe.

Polyinosinic:polycytidylic acid injections.

Polyinosinic:polycytidylic acid (Sigma-Aldrich P9582-5MG) was injected intraperitoneally at 5 ug/kg and monitored every two hours (unless otherwise specified) via a temperature probe.

Cold challenge.

Mice were housed individually for up to 72 hours in 4-8flC. Cold challenge experiments for this chapter allowed ad libitum access to regular chow diet and water. Mice followed the same 12 hr day/12 hr night cycle of the Columbia ICM specific pathogen free housing.

Statistics.

Significance calculations of comparisons between two groups were determined by student's t-test. Significance calculations of multiple group comparisons were determined first by ANOVA and then Bonferroni correction. Key for all significance indications: * = p-value ≤ 0.05; ** = p-value ≤ 0.01; *** = p-value ≤ 0.001.

Figures represent one experiment out of four. Data are not combined to produce the figures or statistics. Error bars represent the standard deviation.

Results

Cold challenge induces a lysosomal biogenesis program in brown adipose tissue.
We hypothesize that lysosomes contribute to regulated thermogenesis. To test this one aspect of our model, we assessed whether cold exposure activates a lysosomal program as our model predicts. We placed 7-8 week old male FVB/NJ mice in the cold (4-8°C) and littermates at room temperature (20-25°C). Mice were sacrificed after 8, 18, or 72 hours at 4-8C. Brown (BAT), subcutaneous (SUBQ) and epididymal (EPID) fat depots were collected for analysis. Using quantitative real-time polymerase chain reaction (qRT) we measured expression of lysosome and thermogenic genes and compared mRNA transcripts levels between room temperature and cold challenged mice. Lysosomal genes were upregulated in 8 and 72 hour time points, but not at 18 hour time point in the BAT (Fig 2.1-2.3). These genes include the lysosomal regulators, transcription factor EB (Tfeb), a positive transcriptional regulator of lysosomes (Sardiello et al. 2009), zinc finger protein with KRAB and a negative regulator SCAB domains (Zkscan3) (Chauhan et al. 2013), lysosomal membrane associated proteins, Lysosomal associated membrane protein 2 (Lamp2), Chloride channel voltage sensitive 7 (Clcn7), and the h subunit of ATPase, H+ transporting, V1 subunit (Atp6v1h). We also found cold-induced upregulation in expression of genes encoding lysosomal hydrolases such as lysosomal acid lipase (Lipa) and glucosamine-6-sulfatase (Gns).

To determine whether cold-induced lysosome gene expression resulted in increases in lysosomal proteins, we measured the LAMP1, LAMP2, and LIPA protein levels in BAT from mice at 4°C and 25°C. (Fig 2.4-2.9). Consistent with the gene expression data, each protein level was increased by cold exposure at 8 and 72 hours, but not at 18 hours. This pattern was further corroborated by the LIPA

activity levels (Fig 2.10) and the immunofluorescence of LAMP1, and perilipin 1 (PLIN1) (Fig 2.11). Together these data demonstrate that cold induces lysosome biogenesis in BAT. They also suggest that there is a bimodal wave of lysosome biogenesis and function, one occurring early and then a second after 24hrs.

Cold challenge does not induce the same lysosomal program in white adipose tissues.

Some white adipose tissue depots contain thermogenic beige adipocytes and their precursors (Giralt and Villarroya 2013). Beige adipocytes, unlike their white counterparts, are rapidly activated during cold exposure. To determine whether cold induces lysosome biogenesis in other fat depots, we examined the lysosomal program in subcutaneous and perigonadal depots. Surprisingly, when we a cold challenge did not induce a lysosomal program (Fig 2.12-2.14) at 8 or 18 hours in gene expression. However, we did notice a modest increase in Lamp2 gene expression level and LIPA activity at 72 hours (Fig 2.14). These data were further corroborated by the LIPA activity assay (Fig 2.15) as well as LAMP1 protein blots (Fig 2.16-2.21). Taken together, this suggests that unlike in BAT, ambient cold temperature that induces thermogenesis does not robustly induce lysosomes in the SUBQ but may have some effect at a more chronic challenge.

Lysosomes are upregulated in non-UCP1 tissues.

We hypothesized that if lysosomes contribute to heat generation directly, then cold challenge would be correlated with an upregulation of lysosomal program even in nonUCP1 tissues. Therefore, we cold challenged 7-8 week old FVB/NJ wildtype mice and isolated various nonUCP1 tissues, liver, kidney, heart, spleen, and skeletal muscle, to determine lysosomal program activation.

Given the upregulation of lysosomes in prolonged cold challenge in BAT, we assessed the lysosomal program in non-UCP1 tissue after 72 hours at 4-8°C to see whether cold challenges regulated lysosome biogenesis in other tissues. While we saw no induction of lysosomal transcripts in kidney (Fig 2.22) and heart (Fig 2.23), the expression of lysosomal genes were to varying degrees induced in slow-twitch skeletal muscle (soleus) (Fig 2.24), spleen (Fig 2.25), and in the liver (Fig 2.26). Consistent with our observations in BAT, a cold challenge that induces nonshivering thermogenesis also induced protein and LIPA activity (except in the soleus) consistent with the induction of gene expression (Fig 2.27-2.35). Both methods corroborated the transcriptional activation of lysosomes in soleus, spleen but not in the liver. Consistent with the qRT findings, the kidney, heart, and the EDL saw no increases or even decreases in lysosome protein and activity. Together, these data demonstrate that lysosomes are activated in a tissue specific manner in UCP1 negative tissues after prolonged exposure to 4-8°C.

Immune cell populations of the spleen are increased after cold challenge.

We were surprised to see activation of lysosome biogenesis and activity in spleens of cold challenged mice. The spleen is a heterogeneous organ that histologically includes the red and white pulp. Red pulp consists of mesenchymal cells, platelets, granulocytes, and red blood cells ("Basic Histology" 2016). White pulp, on the other hand, contains large numbers of B and T lymphocytes, as well as antigen presenting cells (APCs), including macrophage/monocytes populations, and dendritic cells. Our data on cold induced lysosome activation in spleen suggest that immune cells may be directly regulated by cold. To understand how the cell population might change after cold challenge in the whole spleen, we performed qRT-PCR of various immune cell markers. Genes examined included: activated leukocyte cell adhesion molecule (Alcam), cluster of differentiation 2, 4,

8a, 19, 28, and 200r1 (Cd2, Cd4, Cd8a, Cd19, Cd28, Cd200r1), which mark T- and B-lymphocytes, cluster of differentiation 40, 69, (Cd40, Cd69), EGF-like module-containing mucin-like hormone receptor-like 1 (Emr1, or F4/80), endoglin (Eng), which mark monocytes, macrophages, and dendritic cells, and other markers: cluster of differentiation 86 (Cd86, marking activated B-cells, monocytes, macrophages, and dendritic cells), dipeptidyl-peptidase (Dpp4, marking activated T-cells, monocytes, and macrophages), tyrosine protein kinase 1 (Tek1, marking endothelial cells) (Yakimchuk and Konstantin 2016)(Suttles, Jill, and Stout 2009; Cambiaggi et al. 1992; Khazen et al. 2005; Gougos et al. 1992; Waumans et al. 2016; Matteucci and Giampietro 2009). Cold increased *Cd45* expression - a gene expressed by all leukocytes, and increased expression of lymphocyte-specific genes (Fig 2.36). We found modest changes in monocyte, macrophage, and dendritic cell populations as well (Fig 2.36). Furthermore, these data are supported by flow cytometry: Lysotracker (LS) positive cells are increased after cold challenge and are largely F4/80+. Lysosome rich immune cells, as marked by Lysotracker (LS) and CD11B, were more likely to be F4/80 positive, implicating macrophages in the lysosome phenotype (Fig 2.37 and 2.38).

Finally, in the lysosomal storage disease model of LIPA deficiency, we found that these same immune cell changes were blunted (Fig 2.39). This observation suggests that the immune cell population changes in the spleen after cold challenge may be contributing to the lysosome phenotype we observe.

Lysosomes are upregulated in cold challenged poikilotherms.

Whereas homeotherms maintain internal body temperature within a relatively narrow range and typically have complex mechanism to modulate both heat exchange and thermogenesis, poikilotherms, seem to lack complex thermoregulatory systems. Therefore, their internal body temperatures typically

varies more widely. Nonetheless, given the importance of core body temperature in the function of an organism, many poikilotherms have evolved adaptations to drastic variations in environmental temperature (Heath 1970). While poikilotherms lack UCP1 and any known mechanism to uncouple respiration, they do contain lysosomes or vacuoles. Given that modulation and adaptation to variations in the ambient temperature and lysosome degradation are evolutionarily ancient, we hypothesized that cold challenges would activate lysosome program in poikilotherms.

We examined the effects of cold challenge on the poikilothermic, Drosophila melanogaster larvae. Two temperatures were chosen: 20-25°C and 15-18°C. 20-25°C is the optimal functional temperature for Drosophila, while at 15-18°C flies can survive but are impaired in the metabolic and developmental functions. Consistent with our hypothesis, the cold temperature coordinately induced expression of several lysosomal genes, including, lysosome associated membrane protein 1 (lamp1), lipase 1 (lip1), v-type proton ATPase subunit 13 (vha13), and vacoular protein sorting 16 (vps16, responsible for lysosome trafficking) (Pistillo et al. 1998; Tognon et al. 2016; Pulipparacharuvil et al. 2005). In addition, several genes involved in autophagy were also increased (in autophagy-related gene 8 (atg8, an autophagy gene), chloride channel b (clc-b, chloride channel found on Drosophila lysosomes), diabetes- and obesity-regulated gene (dor, an autophagy regulator)(Fig 2.40) (Alemu et al. 2012; Cabrero et al. 2014; Mauvezin et al. 2010). These preliminary results demonstrate that a cold-induced lysosomal transcriptional program is evolutionarily ancient.

Febrile response is correlated with lysosomal genes and protein.

Our data demonstrate that lysosomal programs are activated in response to drops in ambient temperature, consistent with our model that they contribute to regulated thermogenesis. Fevers in mammals also require nonshivering thermogenesis. To test whether a lysosomal program is activated in response to induction of febrile thermogenesis we used two pathogen derived pyrogenic compounds: lipopolysaccharide (LPS) from gram-negative E. Coli and synthetic polyinosinic-polycytidylic acid (poly(I:C)), a mimetic for viral dsRNA. We injected either LPS, Poly(I:C), or saline into lean, young mice and monitored body temperatures every two hours. After injection, we sacrificed the mice and measured expression of lysosomal genes in BAT and spleen. LPS induced the expression of half the lysosomal genes we measured in BAT and about a fifth of the ones in the spleen (Fig 2.41 and 2.42). While LAMP1 protein was not induced by LPS (Fig 2.43 and 2.44), it did increase LIPA activity in BAT (Fig 2.45), though not in spleen (Fig 2.46).

Chloroquine, a lysosomal inhibitor, impairs thermogenic response.

We have found that in response to thermogenic stimuli, lysosomal programs become activated in BAT as well as other tissues not previously implicated in regulated thermogenesis. To investigate whether BAT lysosomal function contributes to thermogenesis and maintenance of body temperature in response to a cold challenge, we treated mice with chloroquine (CQ), a lysosomal inhibitor that reduces the acidification of late endosomes and lysosomes. We injected 3.5 mg/kg of CQ or PBS into the IP of eight week old wildtype FVB mice. Following IP injection of either CQ or phosphate-buffered saline (PBS), the mice were cold challenged at 4°C and temperatures were taken via a rectal probe every two hours. Mice that were injected with CQ dropped temperatures at 4°C while the

littermate controls injected with PBS maintained body temperatures (Fig 2.47) suggesting a functional role for lysosomes in thermogenesis.

To determine whether inhibition of lysosome function with CQ would also impair the febrile response we injected mice with either LPS or poly(I:C). CQ or PBS was injected IP into LPS and poly(I:C) treated animals. Similar to what we observed with cold challenged animals, CQ reduced the body temperature and febrile response of mice in LPS (Fig 2.48) or poly(I:C) (Fig 2.49) treated animals.

Disruption of LIPA function results in cold intolerance.

In our studies of cold-induced and febrile thermogenesis we noted a consistent increase in the expression and activity of Lysosomal acid lipase, the only known acid lipase in mammals. To test whether genetic deficiency in Lipa would impair thermogenesis, we studied the response of Lipa-/- mice to cold challenge and LPS. Lipa-deficient mice are born without gross defects, but starting at 8 weeks of age, begin to lose white adipose tissue and expand BAT. To avoid possible confounding issues, we performed experiments in LIPA KO mice prior to any significant morphological differences in BAT or SUBQ depots between knockouts and wildtypes, at 6-8 weeks of age.

Cold challenge of LIPA-deficient and wildtype littermates showed that LIPA deficiency impairs thermogenesis. Lipa-/- mice rapidly lost body temperatures (Fig 2.50). Tissue analysis revealed that BAT histology (Fig 2.51), lysosomes, mitochondria, and trafficking functions of knockout mice are indistinguishable from those of wildtype mice (Fig 2.52). Taken together, these data suggest that lysosomes are not only correlated with cold challenge, but also necessary for successful defense of body temperature during a cold challenge.

Model.

We found that thermogenic stimuli induce a lysosome program and that inhibiting lysosomes pharmacologically or genetically impairs cold-induced and febrile thermogenesis. The cold-induced lysosomal program is not limited to the classical thermogenic tissue, BAT but is also detectable in spleen, muscle and liver. The transcriptional induction of a lysosomal program in response to a cold challenge also appears to be evolutionarily ancient being induced in Drosophila larvae in addition to mice. Together these findings argue that lysosomes contribute to regulated thermogenesis [Fig 2.53].

Conclusions

As the primary degradative organelles of cells, lysosomes play central roles in the many processes, including processing of antigen for presentation, killing of intracellular pathogens, autophagy and recycling of cellular debris, secreting of hydrolases, and even a process of lysosomal-dependent cell death (Settembre et al. 2013; Ballabio 2016; Appelqvist et al. 2013). Based on the data presented in this chapter, we have discovered that lysosomes also participate in regulated thermogenesis. We demonstrate that lysosomes are upregulated in a tissue-specific manner by thermogenic stimuli, including a cold challenge and inducers of fever. Pharmacological and genetic data further implicate lysosome function as critical for generating heat and maintaining or increasing body temperature.

In mice and humans, most research on nonshivering thermogenesis has centered on UCP1 uncoupling of oxidation in BAT and beige adipose tissue. However, several lines of evidence point to the existence of nonUCP1 dependent mechanisms of NST. Mice that lack UCP1 can maintain body temperature in the face of a cold challenge in a manner that depends on strain background and on the rate at which mice are transitioned to a cold environment. Birds lack a functional homologue of UCP1 yet are homeotherms and are capable of maintaining body temperature in extreme cold, providing indirect evidence for nonUCP1 mechanisms of thermogenesis. Additionally, there exist several plants that are thermogenic. The generation of heat by these organisms are thought to play multiple roles, including attracting insects. To our knowledge none of these studies have focused thermogenesis as part of these adaptive responses.

We posit that lysosomes are part of a thermogenic response of organisms that does not dependent on the expression of UCP1. Indeed, in mice we found that lysosomes were upregulated in some, but not all, of the tissues examined. These

tissues included muscle, the immune cells of the spleen, and possibly liver. Both kidneys and heart showed no induction of lysosome activity following cold exposure. These observations support our overarching model that lysosomes are part of a conserved mechanism to generate heat.

Likewise, this inclusive model, unlike the current exclusive definition of nonshivering thermogenesis, may explain our peculiar observation regarding poikilotherms. As animals without well-functioning cellular pathways that regulate body temperature, poikilotherms often rely on behavioral adaptations, i.e., moving from an area of one temperature to an area of another temperature. Intriguingly, poikilotherms have evolved to survive with turbulent, constantly fluctuating internal body temperatures as a result of a highly adaptable set of enzymes that can function across a range of temperatures, unlike those of homeotherms. Other adaptations may exist that have yet to be described. We believe one such adaptation could be lysosomal related. Our data establishing a correlation between lysosomal gene transcription and cold exposure in poikilotherms support this possibility. As such, this evolutionarily conserved phenomenon further points to the crucial nature of lysosomes in body temperature homeostasis.

Body temperature regulation and thermogenesis are not limited to the context of cold challenge. The phenomenon of febrile response is currently explained by vasodilation and BAT activation. However, this rather constrained explanation fails to accommodate fever induction in UCP1 deficient mice (Okamatsu-Ogura et al. 2007). Given our model, we tested whether lysosomes may be one of the missing players. Our LPS and Poly(I:C) injections support this proposal. Both fever-inducing agents activate lysosomal gene transcription, protein expression,

and lysosomal acid lipase activity in BAT, the spleen, and perhaps in even liver during febrile response.

Additionally, this chapter demonstrated that lysosomes are not only associated with, but also functionally required for thermogenic responses. Both pharmacological and genetic inhibition of lysosomal function disrupted cold tolerance. A BAT specific injection of chloroquine, which accumulates in adipose tissue, showed cold sensitivity, while lysosomal acid lipase deficiency resulted in a much more severe cold intolerant phenotype. These data indicate the importance of full lysosomal function in maintaining body temperature. In further support of these observations, fever induction of LPS and poly(I:C) both are blunted in chloroquine injected and LIPA deficient mice. Such evidence support our hypothesis and suggest that lysosomes, and particularly lysosomal acid lipase, are important in pyrexia.

Limitations and concerns.

Nonspecific effects.

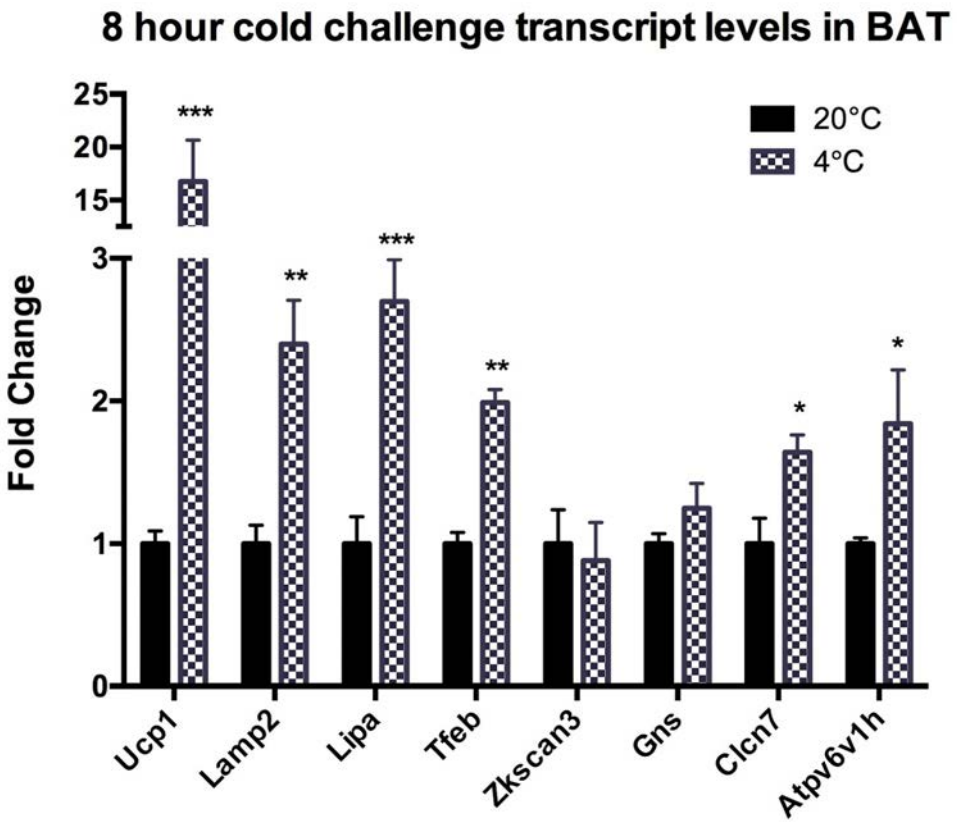
Lysosomes are multifunctional organelles. Cold exposure is a major whole body stress and may easily indirectly involve lysosomes as a result. While our chloroquine and LIPA deficient models suggest that there is causative mechanism, general cellular disruptions in an important cellular machinery may non-specifically impair key cellular functions. In the case of BAT, this could involve UCP1 uncoupling. However, in our studies of *Lipa*- deficient mice we did not observe any gross derangements to BAT, nor is acute chloroquine treatment known to cause broad non-specific impairment in cellular functions.

Cell specificity.

The observed lysosomal induction with thermogenesis is specific to certain tissues. However, these tissues, including the liver, spleen and the brown adipose tissue, are heterogeneous, with a number of cell types. This heterogeneity prevents us from understanding the true source of the lysosomal phenotype. Unfortunately, isolating the various cell populations within these tissue types adds an additional confounding variable. Isolation protocols are time consuming and require digestions, centrifugation, and various other handlings. Additional phenotypes could be added, while others could be lost, making both negative and positive results less reliable and interpretable.

Figures

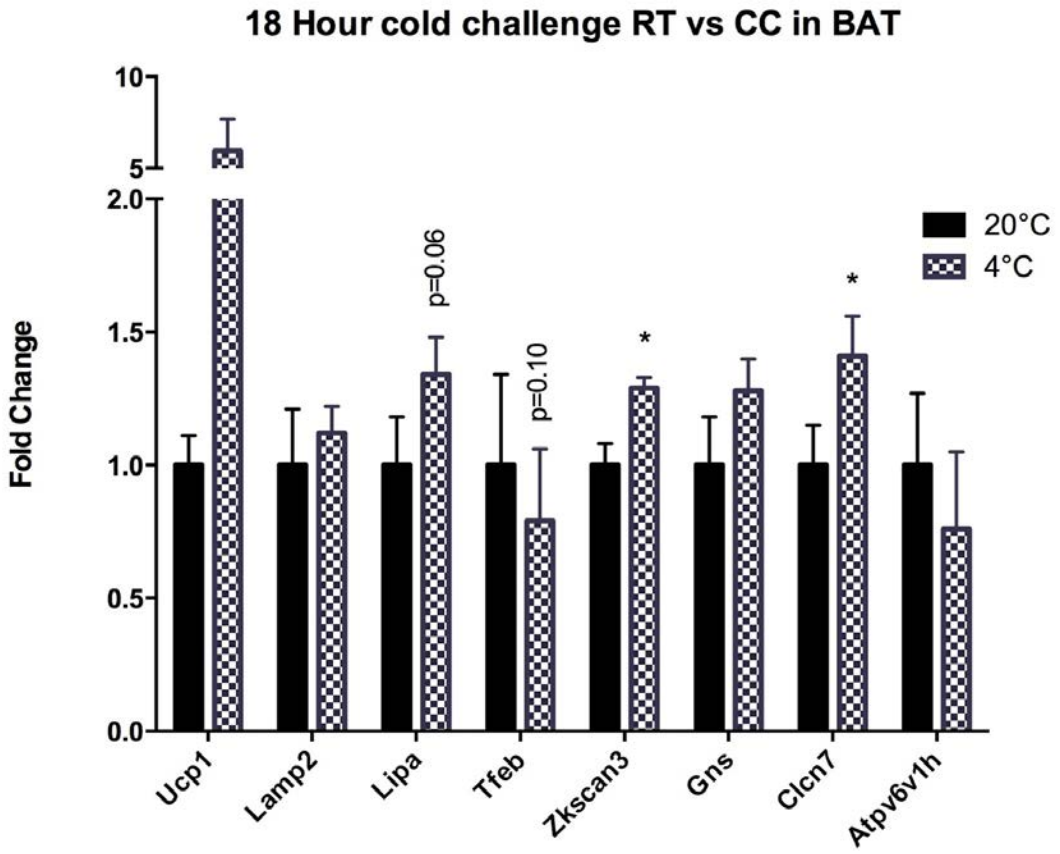
Figure 2.1



Eight hour cold challenge increases lysosomal associated gene transcription in BAT. Transcriptional levels of lysosomal associated genes in whole BAT depots of mice either in 20 C or 4 C after eight hours.

n = 6

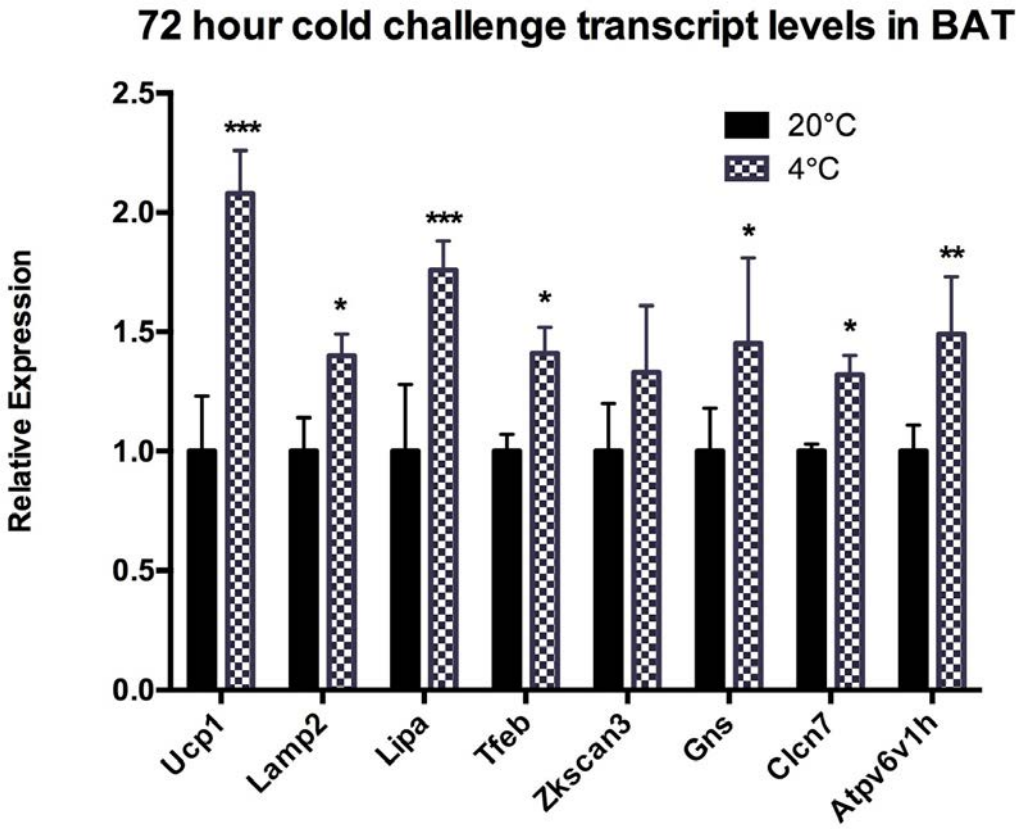
Fig 2.2



Eighteen hour cold challenge increases certain lysosomal associated gene transcription in BAT. Transcriptional levels of lysosomal associated genes in whole BAT depots of mice either in 20 C or 4 C after eighteen hours.

n = 6

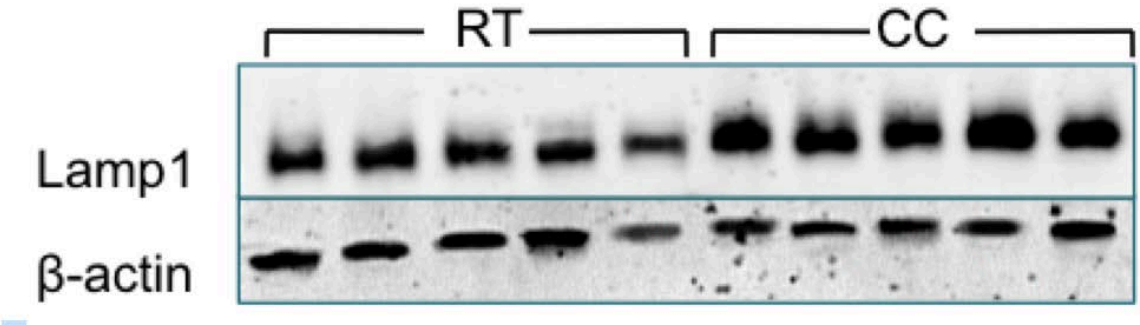
Figure 2.3



72 hour cold challenge increases lysosomal associated gene transcription in BAT. Transcriptional levels of lysosomal associated genes in whole BAT depots of mice either in 20 C or 4 C after eight hours.

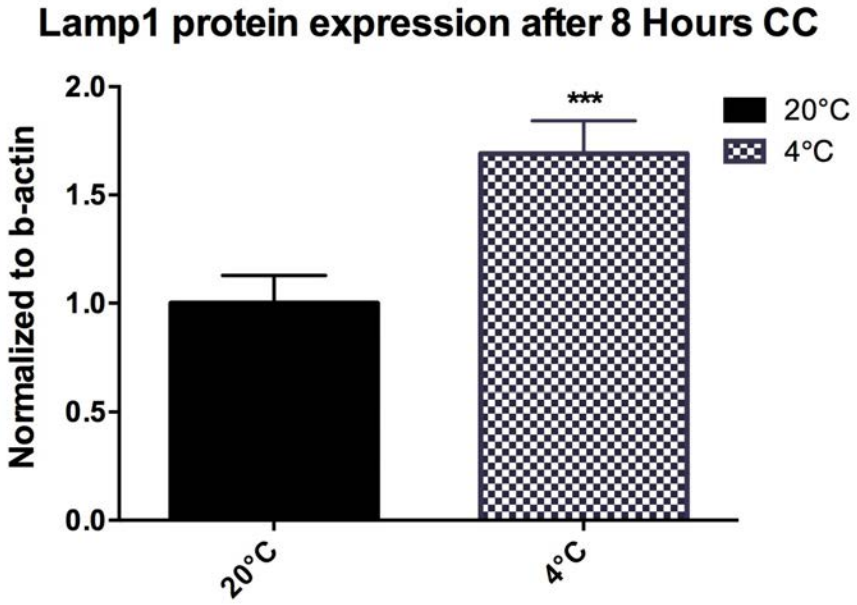
n = 6

Figure 2.4



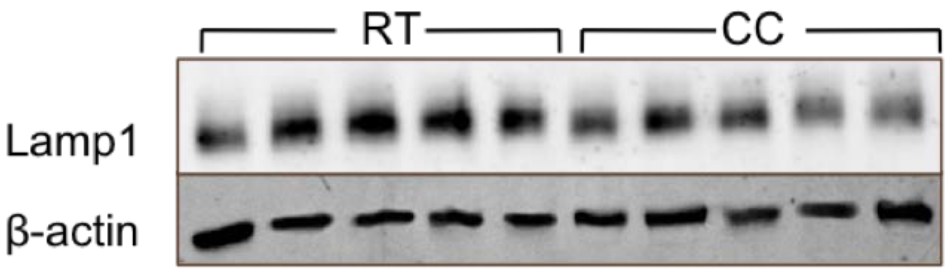
Eight hour cold challenge leads to an increase in LAMP1 protein expression in BAT. Immunoblot of LAMP1 and b-ACTIN in BAT after eight hours in the cold. RT: room temperature exposed mice; CC: cold challenged mice.

Figure 2.5



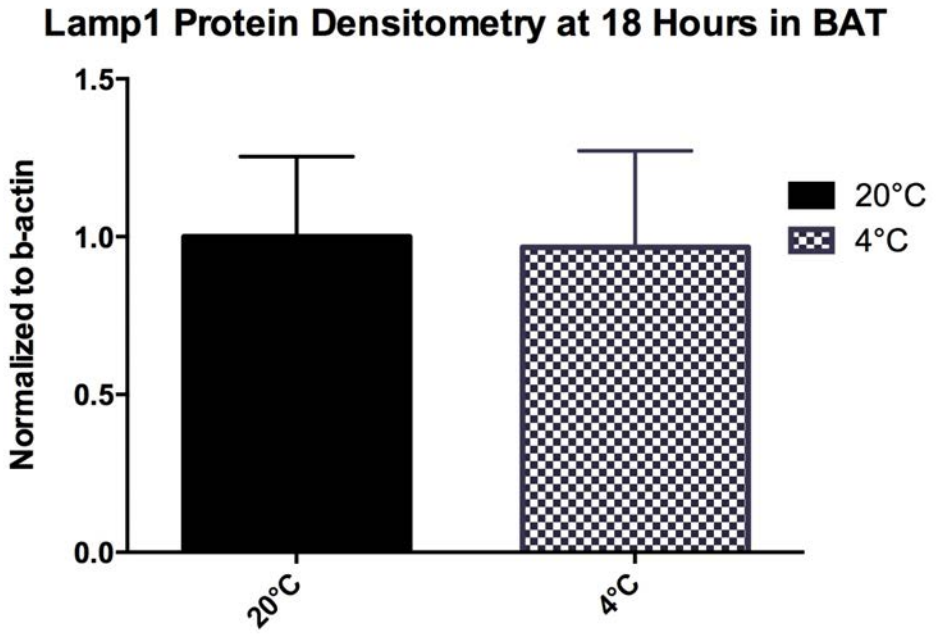
Lamp1 protein expression increases in cold challenged wildtype mice after eight hours. Densitometry of LAMP1 protein expression in BAT depots of mice either in 20 C or 4 C after eight hours.

Figure 2.6



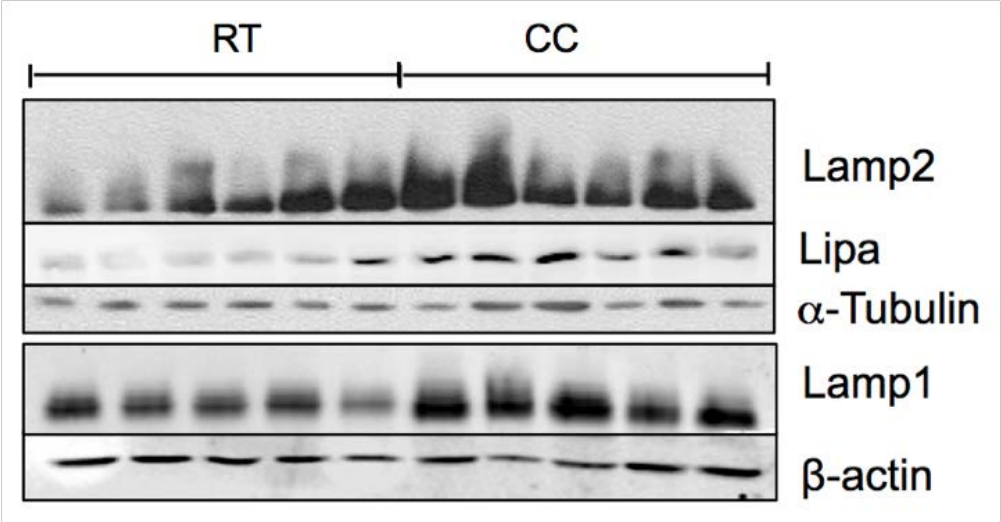
Eighteen hour cold challenge leads to an increase in LAMP1 protein expression in BAT. Immunoblot of LAMP1 and b-ACTIN in BAT after 18 hours in the cold. RT: room temperature exposed mice; CC: cold challenged mice.

Figure 2.7



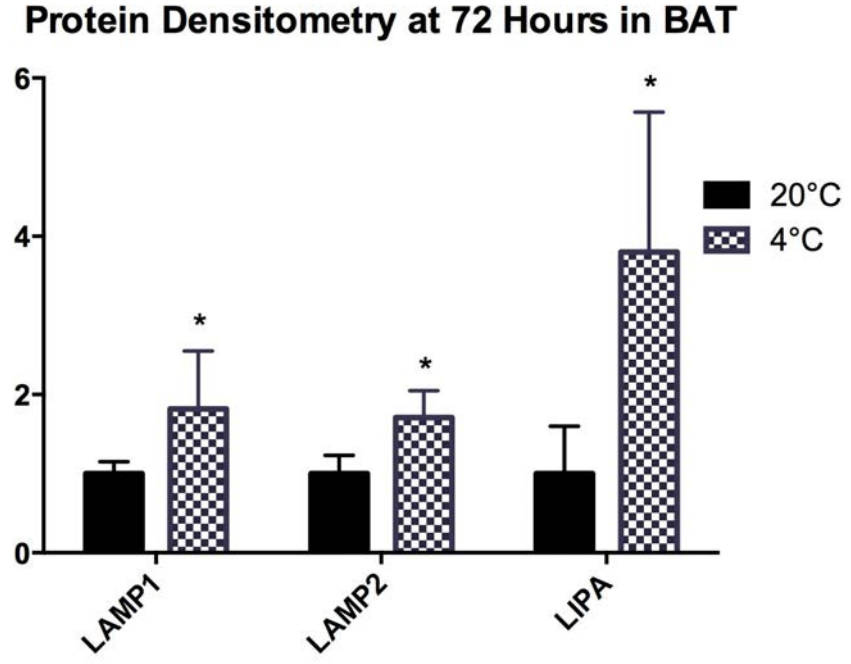
LAMP1 protein expression does not change in cold challenged wildtype mice after eighteen hours. Densitometry of LAMP1 protein expression in BAT depots of mice either in 20 C or 4 C after eighteen hours.

Figure 2.8



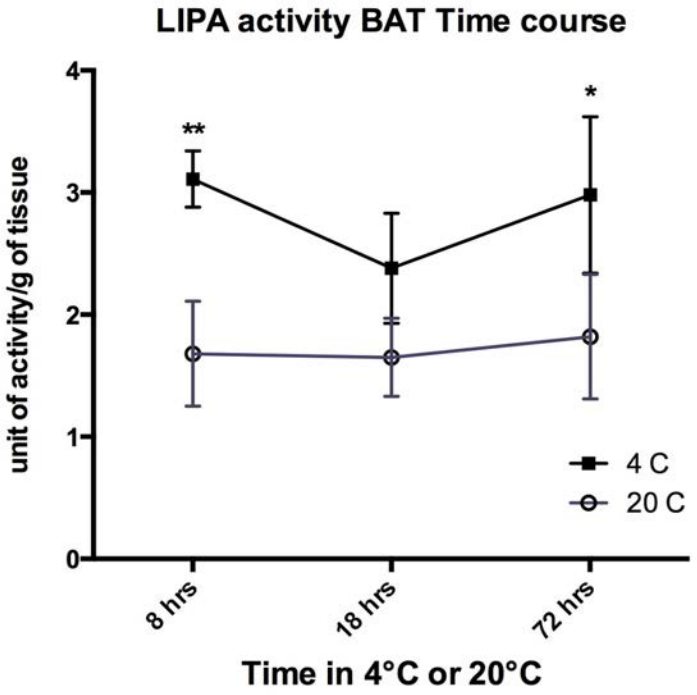
72 hour cold challenge leads to an increase in expression of various lysosomal associated proteins in BAT. Immunoblot of LAMP2, LIPA, LAMP1, a-TUBULIN and b-ACTIN in BAT after 72 hours in the cold. RT: room temperature exposed mice; CC: cold challenged mice.

Figure 2.9



Lysosomal associated protein expression increases in 72 hours in BAT after cold challenge. Densitometry of LAMP1, LAMP2, LIPA protein expression in BAT depots of mice either in 20 C or 4 C after 72 hours.

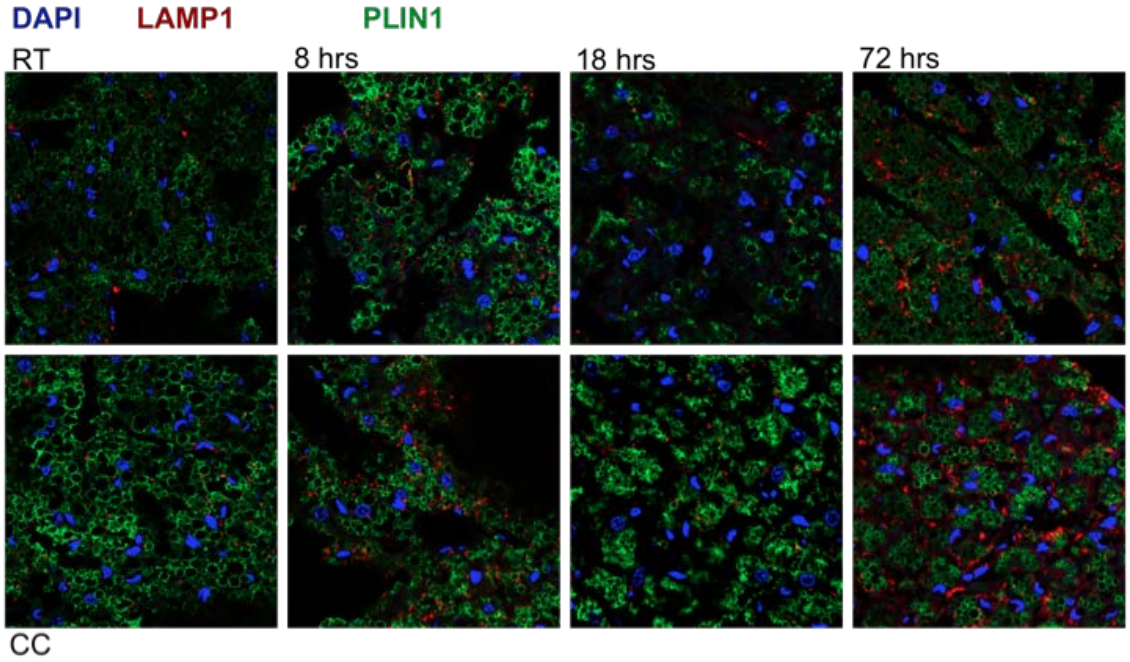
Figure 2.10



LIPA activity is increased in the BAT of cold exposed wildtype mice at 8 hrs and 72 hrs.
LIPA activity in BAT depots measured by 4-MUO activity at 8, 18, and 72 hours at 4 C.

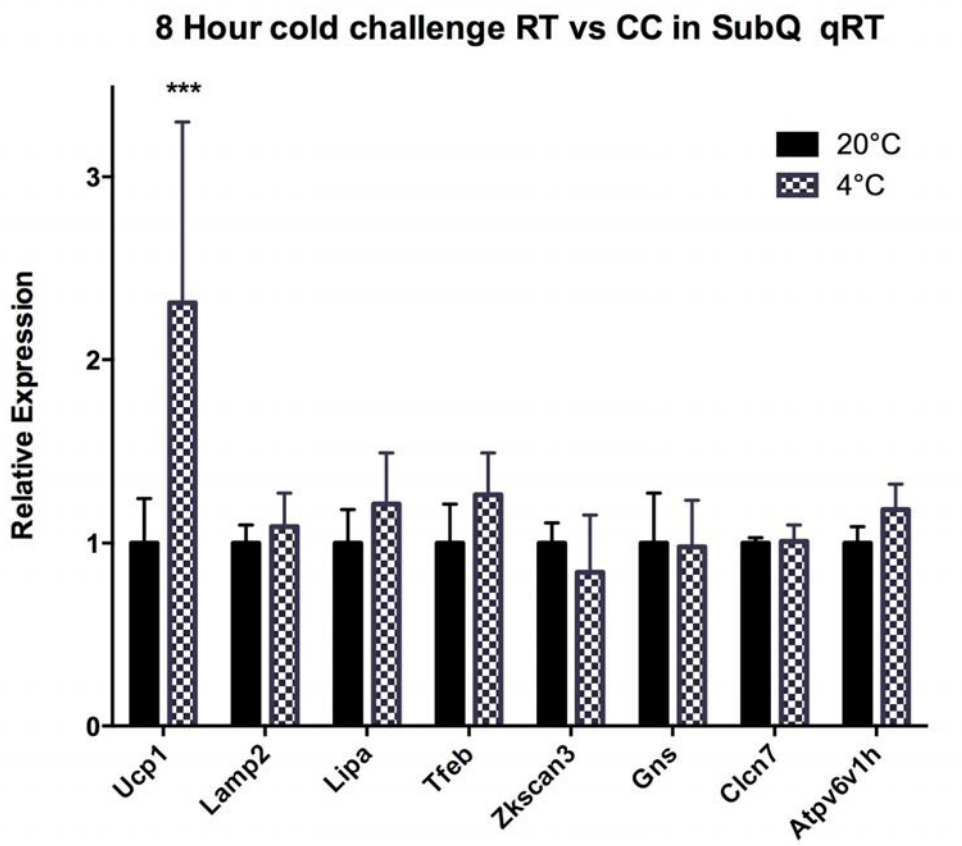
n = 5

Figure 2.11



Increased LAMP1 expression in BAT tissue sections after cold exposure. Immunofluorescence of DAPI (nucleus), LAMP1 (lysosomes), and PLIN1 (lipid droplets) in BAT frozen tissue sections after 8, 18, or 72 hrs in either room temperature or cold. RT: room temperature exposed mice; CC: cold challenged littermates. **n = 6**

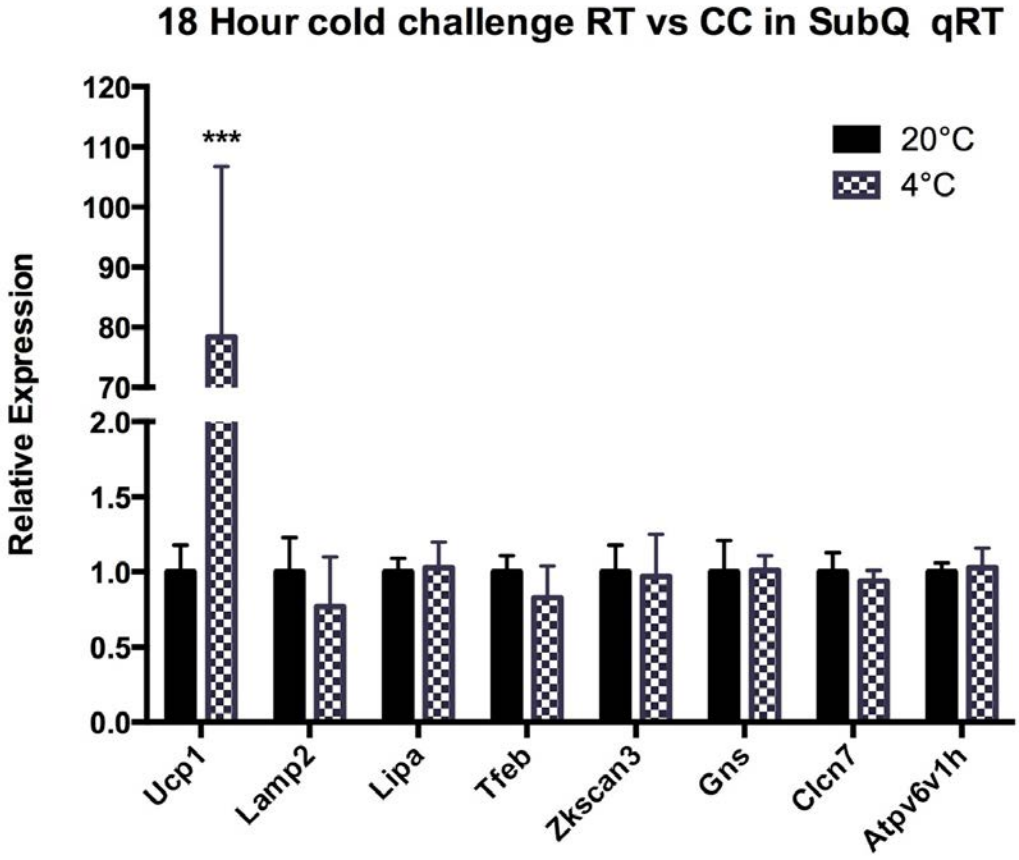
Figure 2.12



Eight hour cold challenge does not alter lysosomal associated gene transcription in SubQ. Transcriptional levels of lysosomal associated genes in whole SubQ depots of mice either in 20 C or 4 C after eight hours.

n = 6

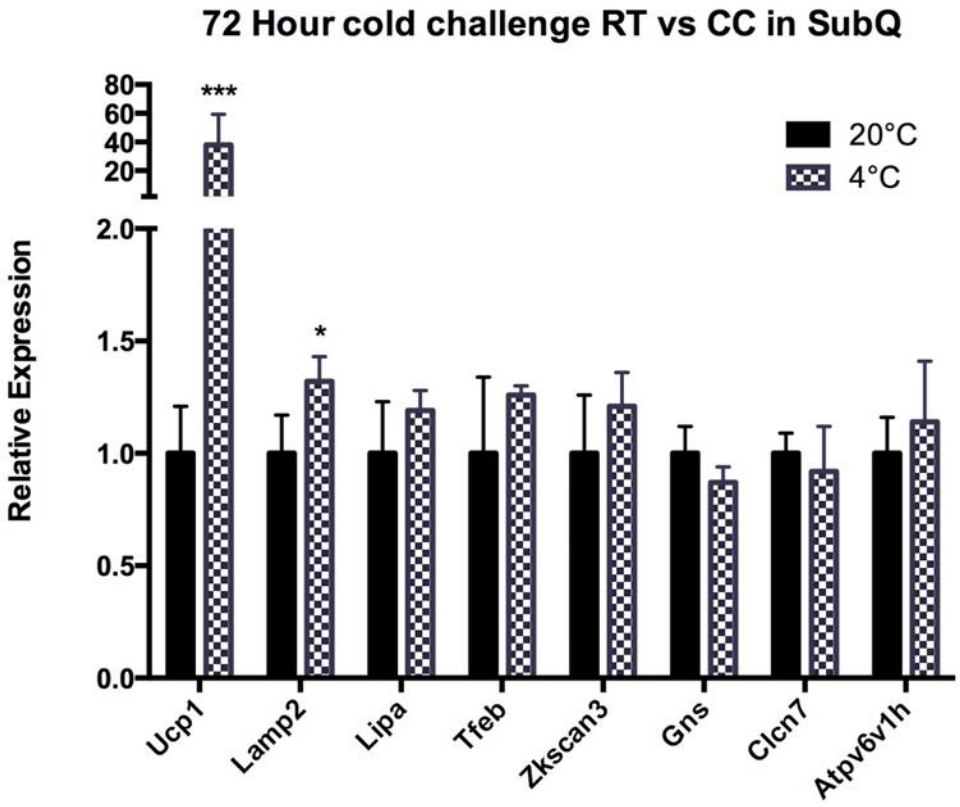
Figure 2.13



Eighteen hour cold challenge does not alter lysosomal associated gene transcription in SubQ. Transcriptional levels of lysosomal associated genes in whole SubQ depots of mice either in 20 C or 4 C after eighteen hours.

n = 6

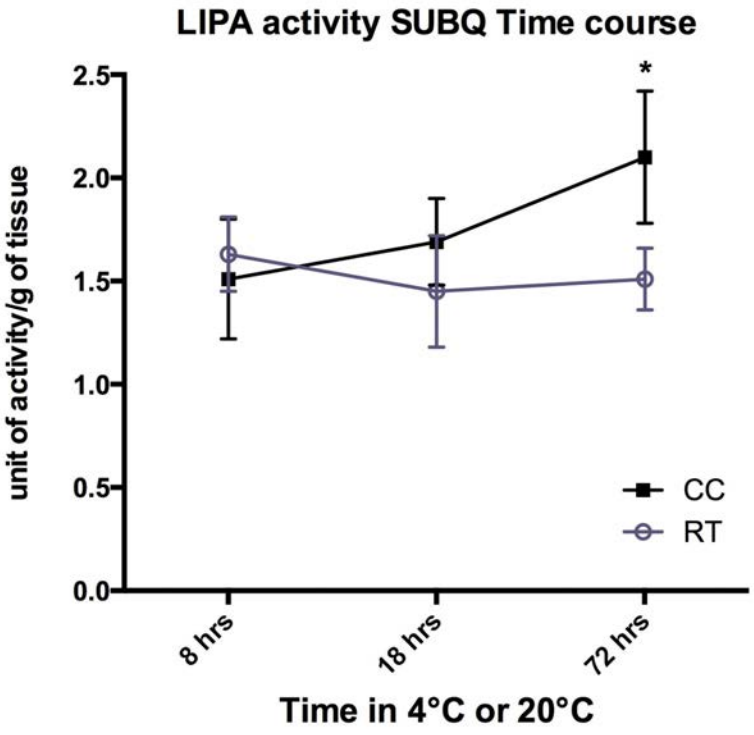
Figure 2.14



72 hour cold challenge increases only LAMP2 gene transcription in SubQ. Transcriptional levels of lysosomal associated genes in whole SubQ depots of mice either in 20 C or 4 C after 72 hours.

n = 6

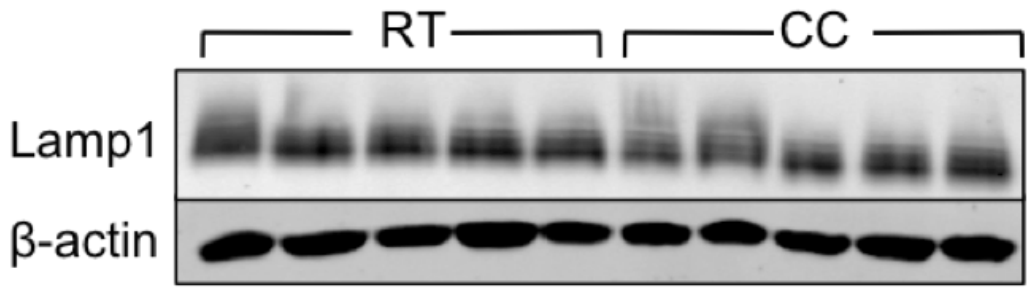
Figure 2.15



LIPA activity is increased in the SUBQ of cold exposed wildtype mice at 72 hrs. LIPA activity in BAT depots measured by 4-MUO activity at 8, 18, and 72 hours at 4 C.

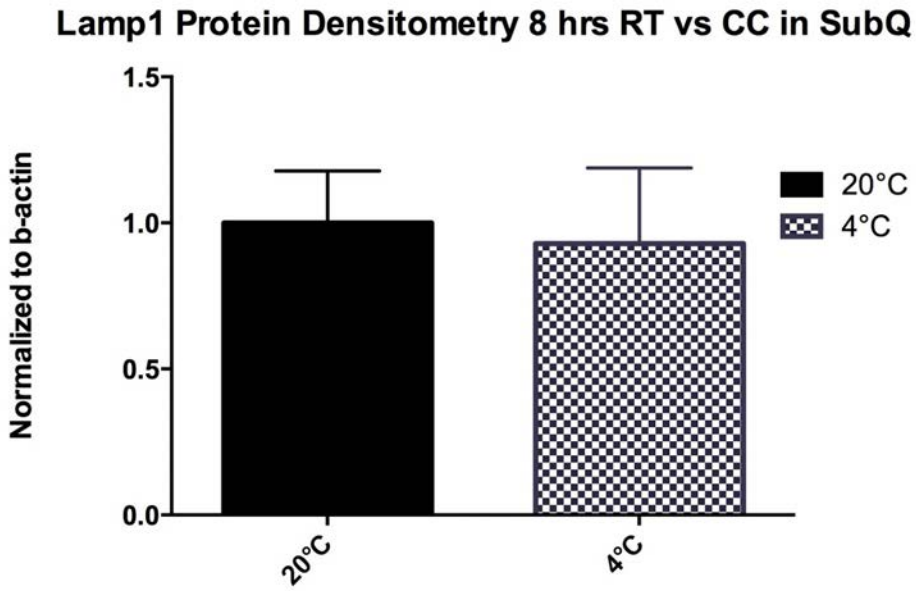
n = 5

Figure 2.16



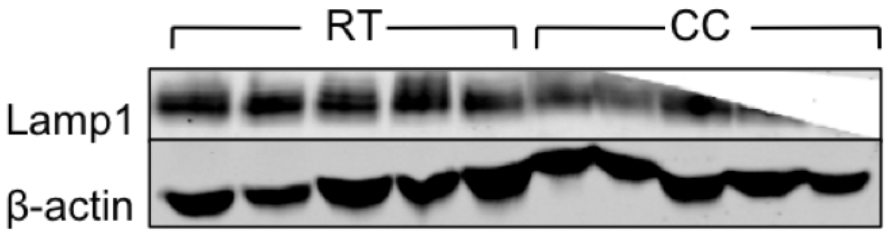
Eight hour cold challenge does not alter LAMP1 protein expression in SUBQ. Immunoblot of LAMP1 and b-ACTIN in SUBQ after eight hours in the cold. RT: room temperature exposed mice; CC: cold challenged mice.

Figure 2.17



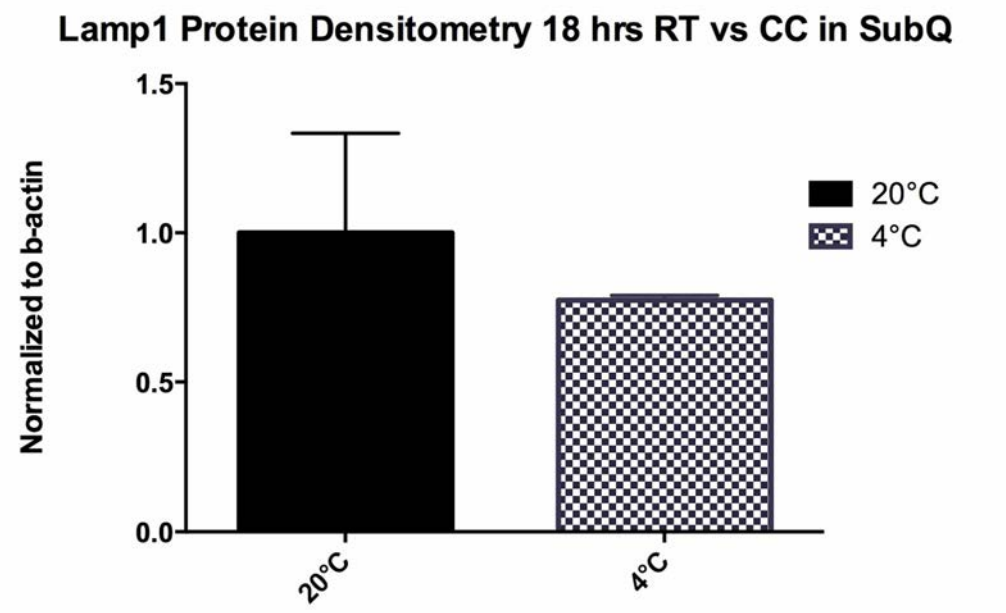
LAMP1 protein expression does not change in SubQ cold challenged wildtype mice after eight hours. Densitometry of LAMP1 protein expression in SubQ depots of mice either in 20 C or 4 C after eight hours.

Figure 2.18



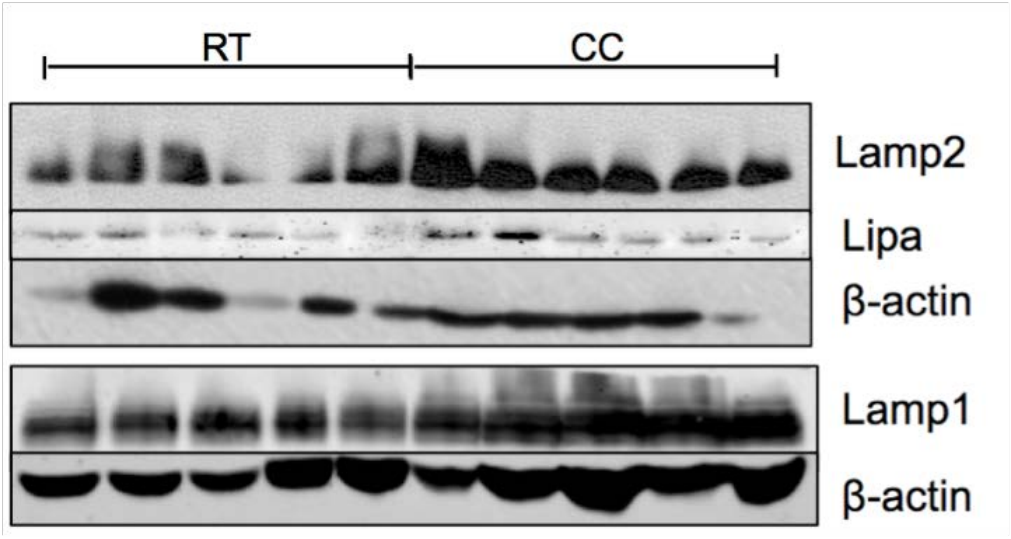
18 hour cold challenge does not alter LAMP1 protein expression in SUBQ. Immunoblot of LAMP1 and b-ACTIN in SUBQ after 18 hours in the cold. RT: room temperature exposed mice; CC: cold challenged mice.

Figure 2.19



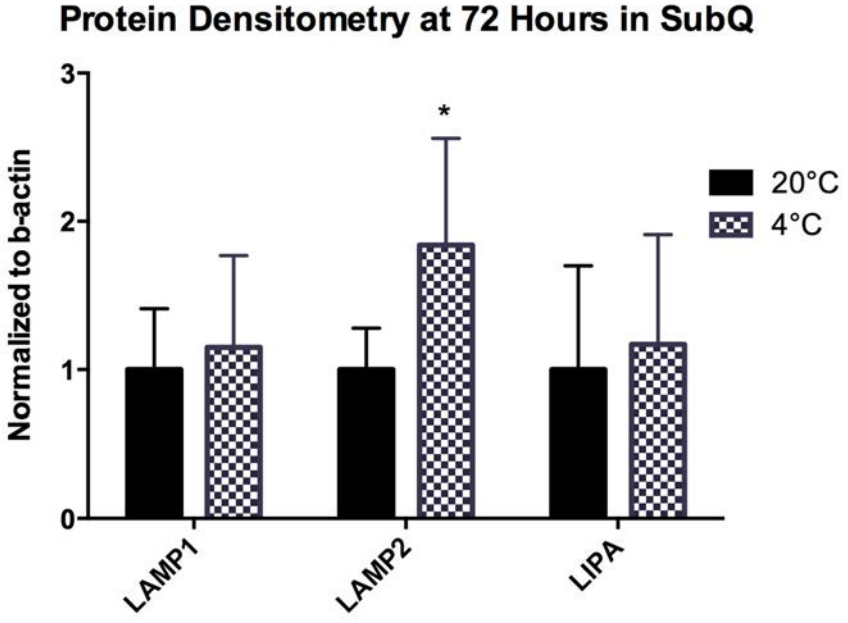
LAMP1 protein expression does not change in SubQ cold challenged wildtype mice after eighteen hours. Densitometry of LAMP1 protein expression in SubQ depots of mice either in 20 C or 4 C after eighteen hours.

Figure 2.20



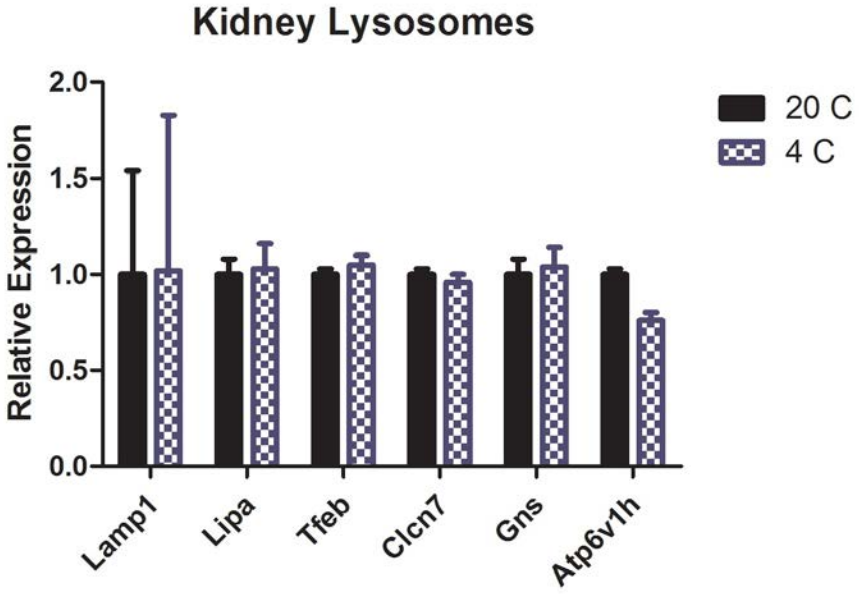
72 hour cold challenge leads to an increase in LAMP2 expression in SUBQ. Immunoblot of LAMP2, LIPA, LAMP1, a-TUBULIN and b-ACTIN in SUBQ after 72 hours in the cold. RT: room temperature exposed mice; CC: cold challenged mice.

Figure 2.21



Lysosomal associated protein expression increases in 72 hours in SubQ after cold challenge. Densitometry of LAMP1, LAMP2, LIPA protein expression in SubQ depots of mice either in 20 C or 4 C after 72 hours.

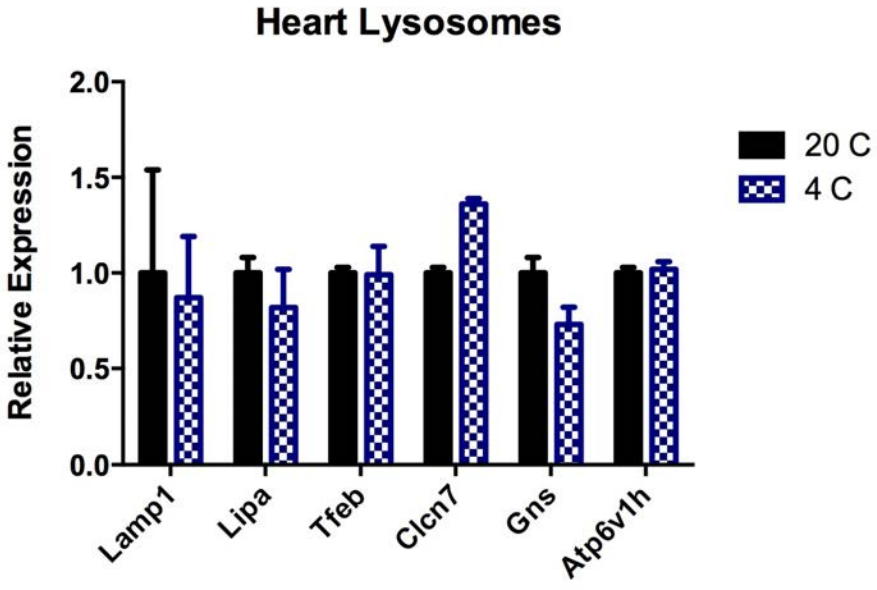
Figure 2.22



Cold challenge does not alter lysosomal associated gene transcripts. Whole kidney samples from cold challenged, wildtype, FVB/J mice showed no changes in the transcription of lysosomal associated genes, as measured by qRT-PCR.

n = 5

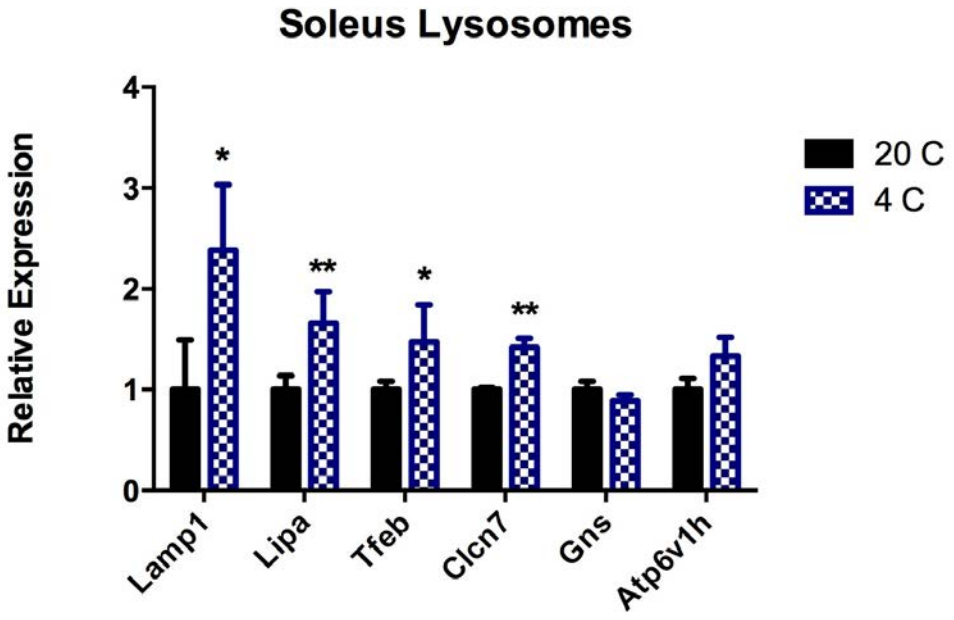
Figure 2.23



Cold challenge does not alter lysosomal associated gene transcripts in the heart. Whole heart samples from cold challenged, wildtype, FVB/J mice showed no changes in the transcription of lysosomal associated genes, as measured by qRT-PCR.

n = 5

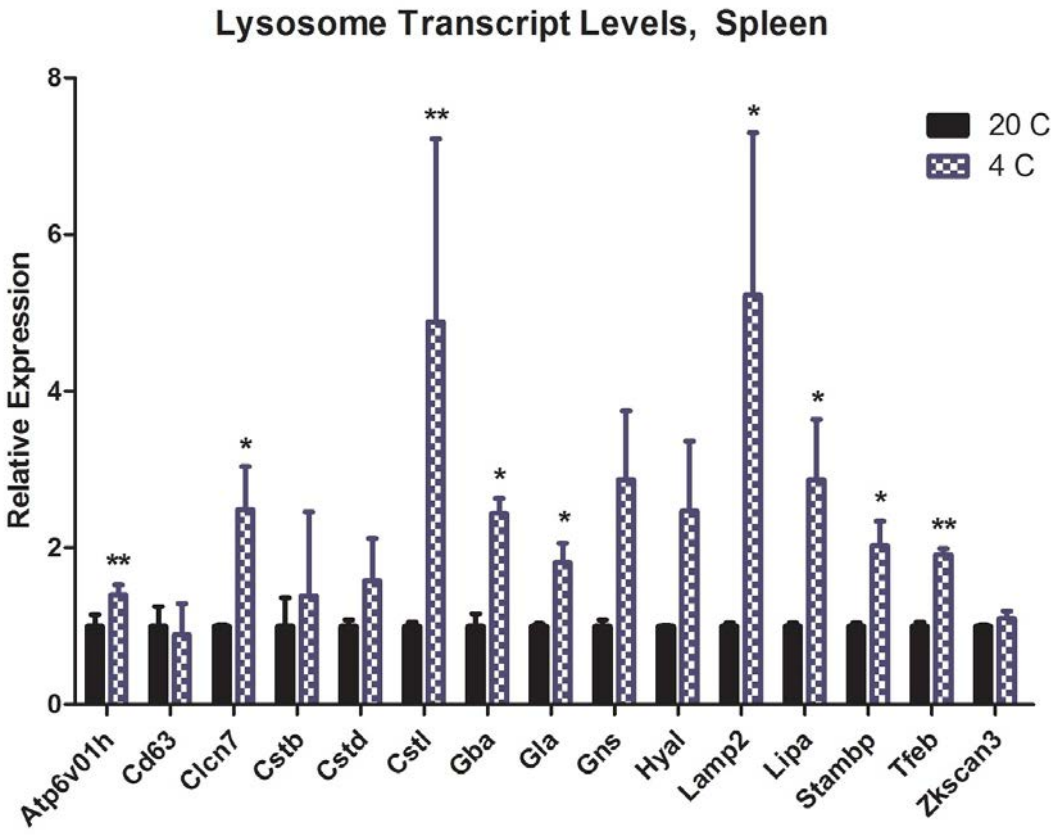
Figure 2.24



Cold challenge alters lysosomal associated gene transcripts in the heart. Soleus samples from cold challenged, wildtype, FVB/J mice showed inductions in the transcription of lysosomal associated genes, as measured by qRT-PCR.

n = 5

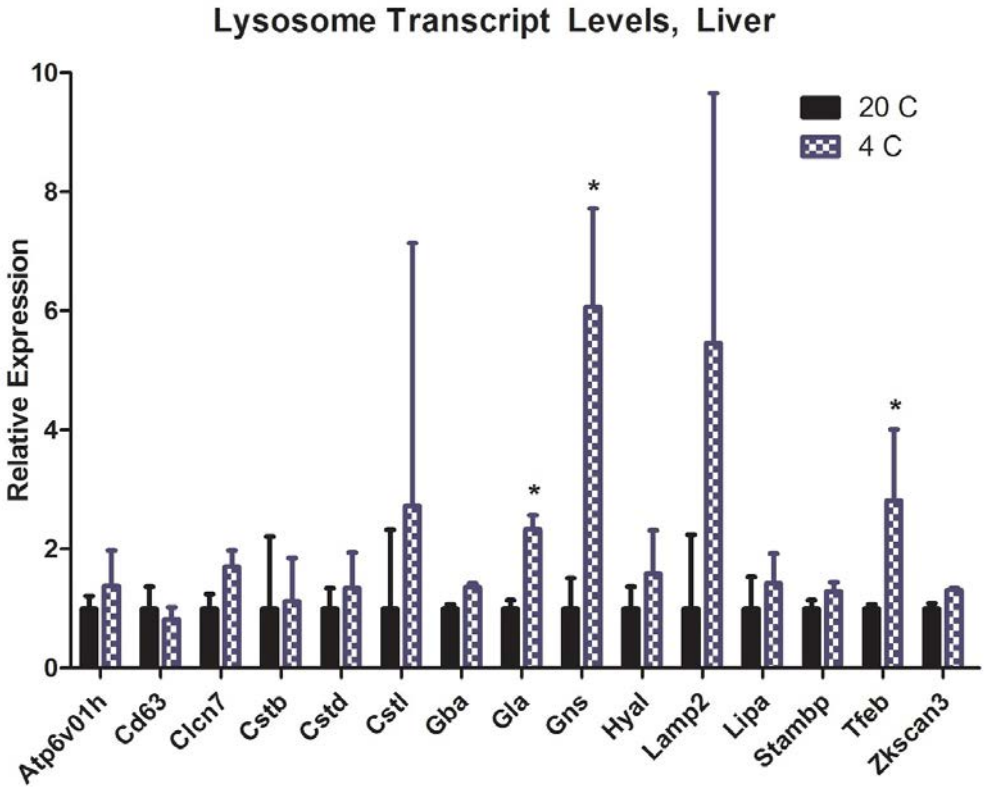
Figure 2.25



Cold challenge increases certain lysosomal associated gene transcripts. Whole spleen samples from cold challenged, wildtype, FVB/J mice showed increased transcription of lysosomal associated genes, as measured by qRT-PCR.

n = 5

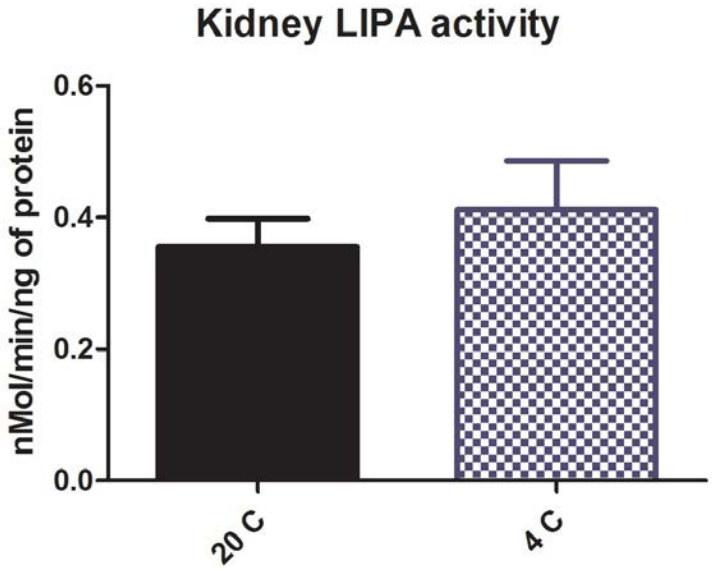
Figure 2.26



Cold challenge increases certain lysosomal associated gene transcripts. Liver tissue samples from cold challenged, wildtype, FVB/J mice showed increased transcription of lysosomal associated genes, as measured by qRT-PCR.

n = 5

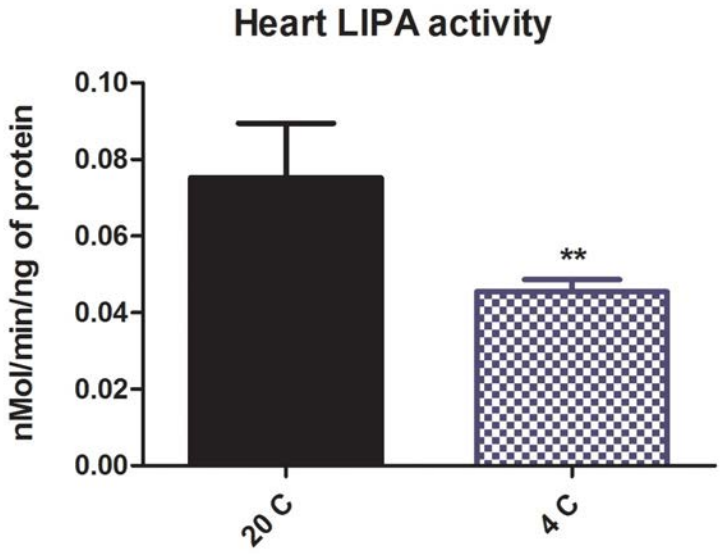
Figure 2.27



Cold challenge does not alter LIPA activity in the kidney. Whole kidneys from cold challenged, wildtype, FVB/J male mice showed no change in LIPA activity, as measured by the 4-MUO assay.

n = 5

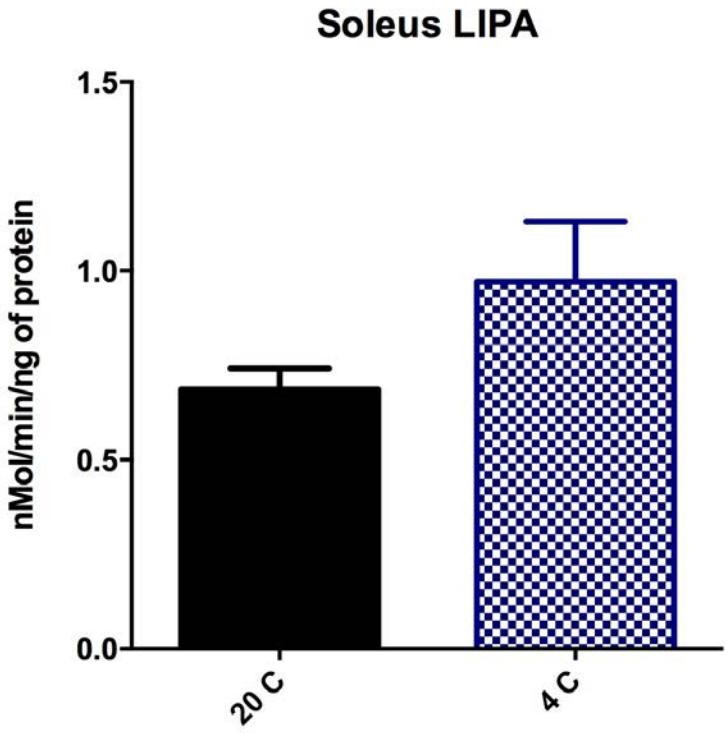
Figure 2.28



Cold challenge decreases LIPA activity in the heart. Whole hearts from cold challenged, wildtype, FVB/J male mice showed a modest decrease in LIPA activity, as measured by the 4-MUO assay.

n = 5

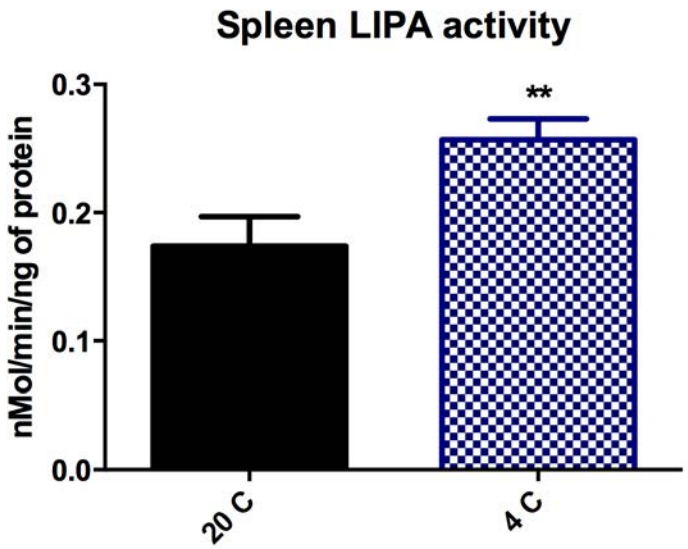
Figure 2.29



Cold challenge does not alter LIPA activity in the soleus Soleus tissue from cold challenged, wildtype, FVB/J male mice showed no changes in LIPA activity, as measured by the 4-MUO assay.

n = 5

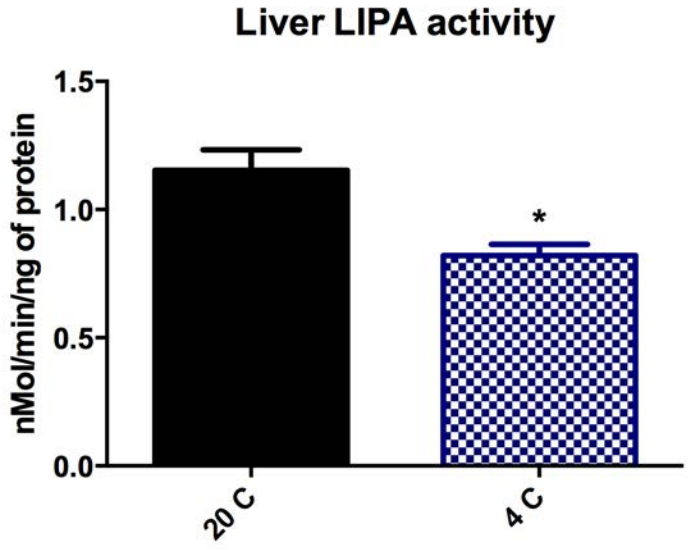
Figure 2.30



Cold challenge increases LIPA activity in the spleen. Spleen tissue samples from cold challenged, wildtype, FVB/J male mice showed an increase in LIPA activity, as measured by the 4-MUO assay.

n = 5

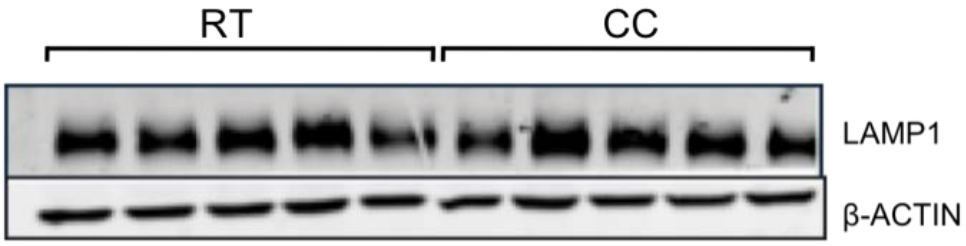
Figure 2.31



Cold challenge decreases LIPA activity in the liver. Liver tissue samples from cold challenged, wildtype, FVB/J male mice showed a small decrease in LIPA activity, as measured by the 4-MUO assay.

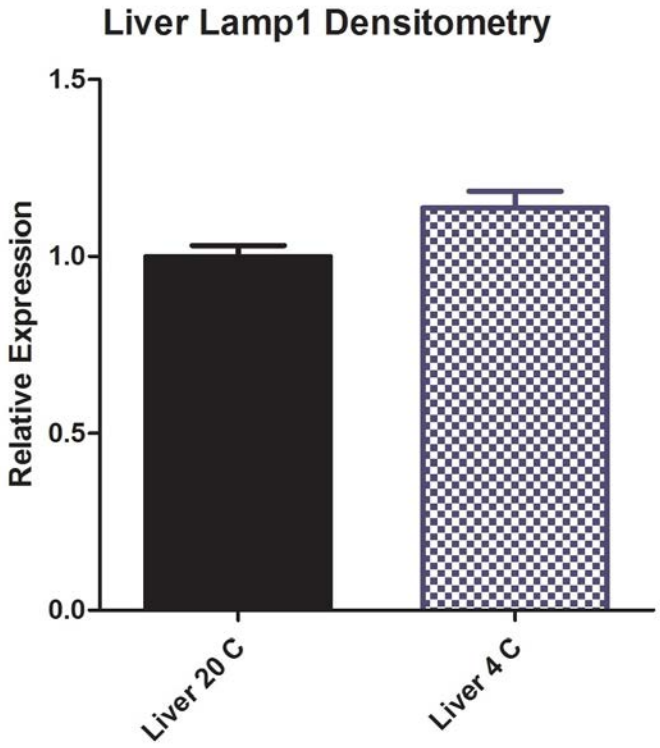
n = 5

Figure 2.32



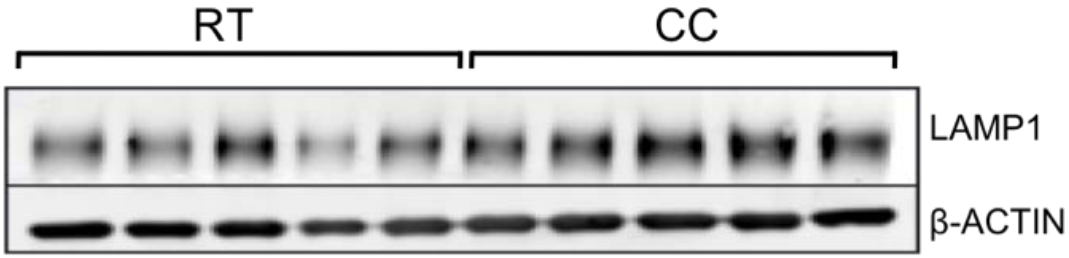
Liver LAMP1 protein expression does not change after cold exposure. LAMP1 immunoblot from liver tissue after cold challenge in wildtype male mice. RT: room temperature exposed mice; CC: cold challenged male littermates.

Figure 2.33



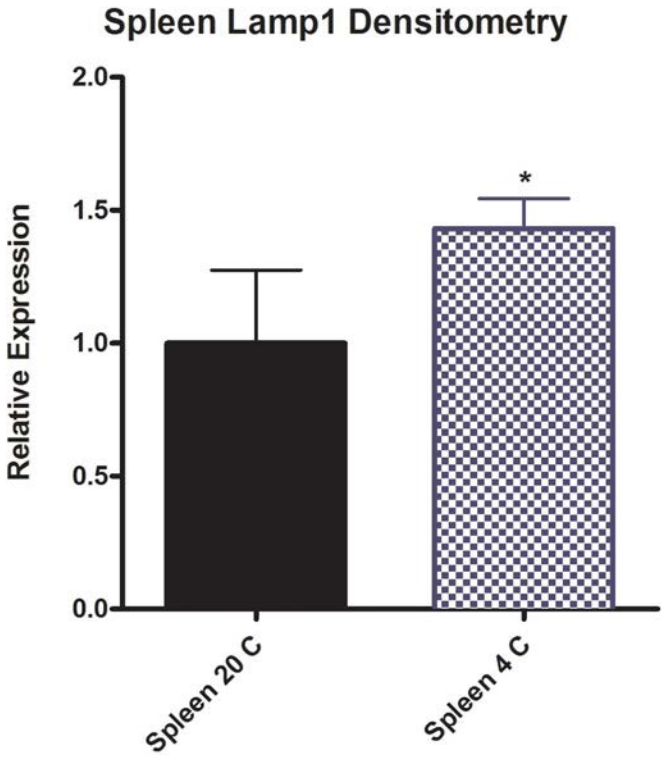
Cold challenge does not alter LAMP1 expression in the liver. Densitometry calculated from Image-J indicates little change in LAMP1 protein expression in the liver.

Figure 2.34



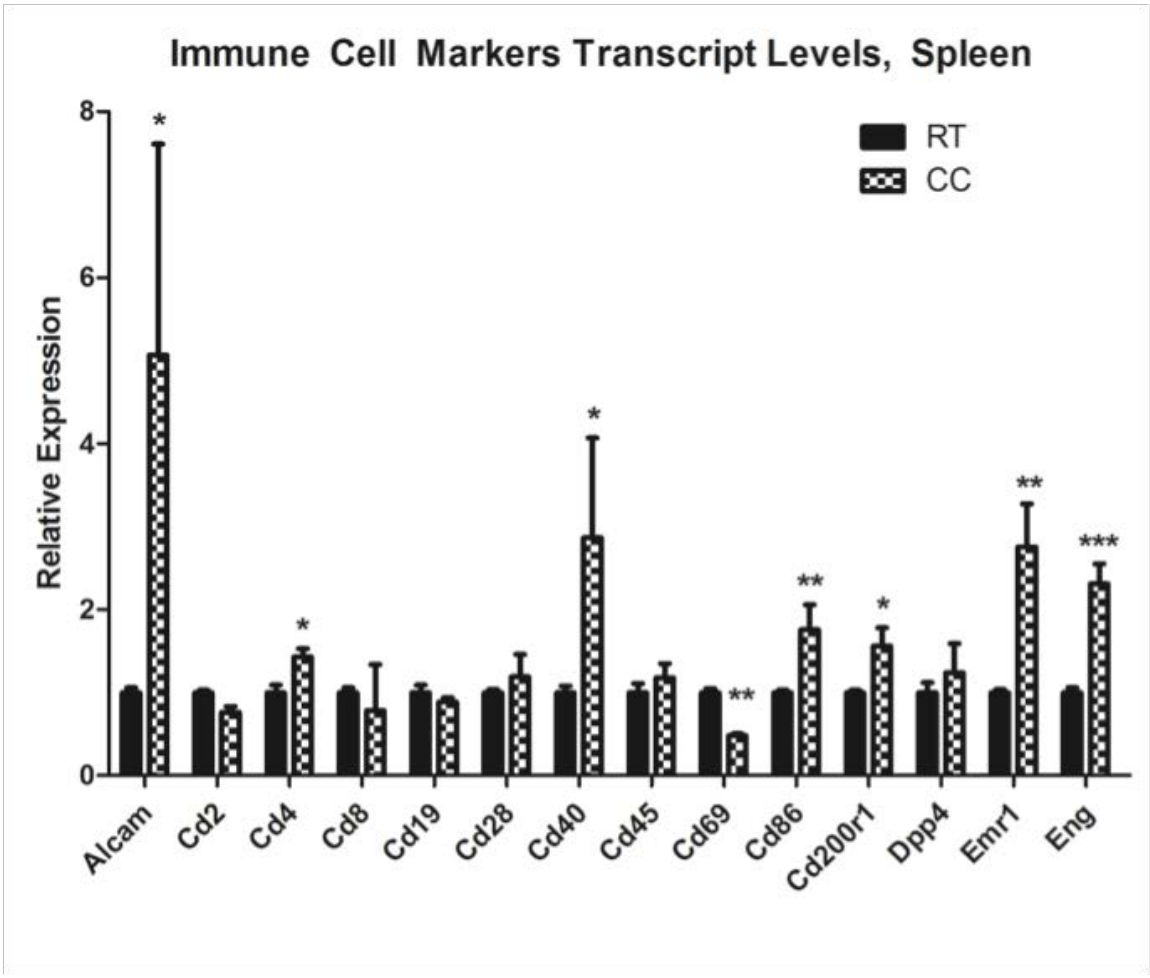
Spleen LAMP1 protein expression increases after cold exposure. LAMP1 immunoblot from whole spleen after cold challenge in wildtype male mice. RT: room temperature exposed mice; CC: cold challenged male littermates.

Figure 2.35



Cold challenge increases LAMP1 expression in the spleen. Densitometry calculated from Image-J indicates an increse in LAMP1 protein expression in the spleen.

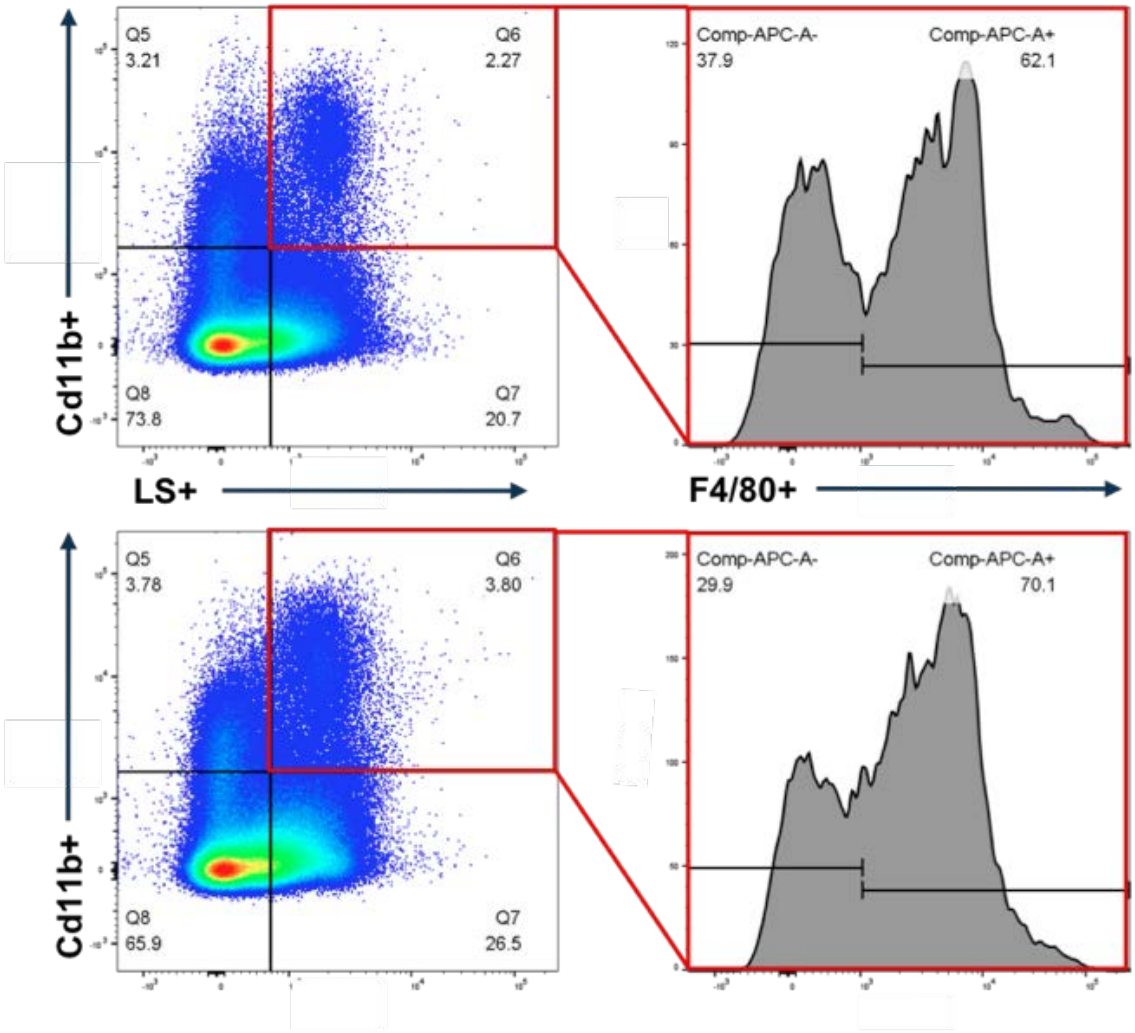
Figure 2.36



Increased immune cell markers in the spleen after cold challenge. Quantitative real time PCR of various immune cell markers in the spleen after cold challenge. RT: room temperature exposed mice; CC: cold challenged male littermates. **n = 5**

Figure 2.37

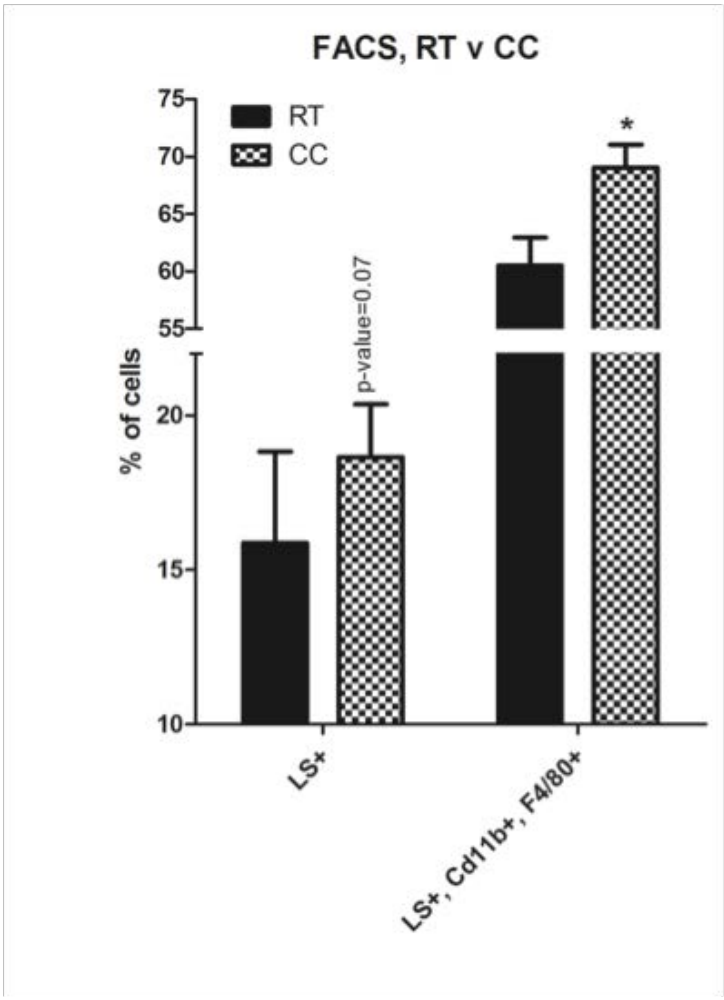
ROOM TEMPERATURE



COLD CHALLENGE

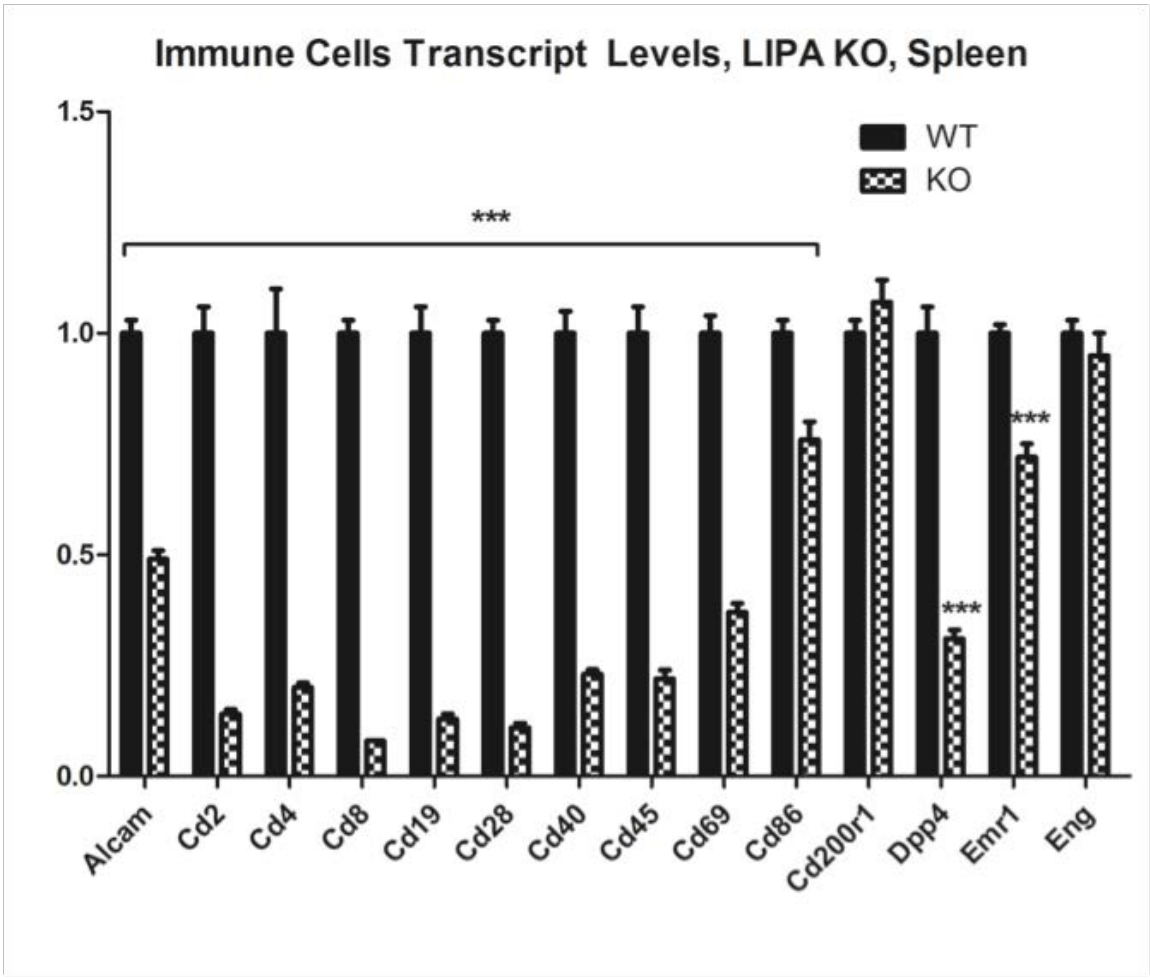
Cd11b+, LS+ cells are more likely to be F4/80+ after cold exposure. Flow cytometry data of Cd11b+, LS+, and F4/80+ cells from splenocyte isolation after cold challenge. Top: Room temperature exposed male mice; Bottom: Cold challenged male littermates. **n = 5**

Figure 2.38



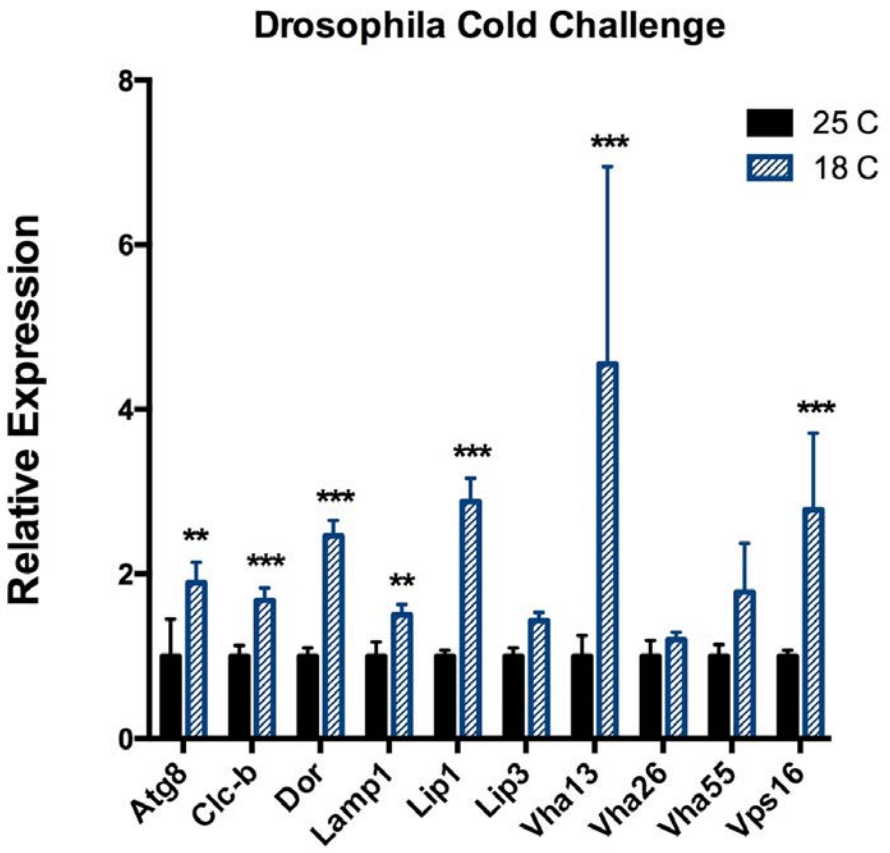
Quantification of gating strategies from Figure 2.37. Flow cytometry data of Cd11b+, LS+, and F4/80+ cells from splenocyte isolation after cold challenge. Top: Room temperature exposed male mice; Bottom: Cold challenged male littermates.

Figure 2.39



Decreased immune cell markers in spleen of LIPA KO mice after cold challenge compared wildtype siblings. Quantitative real-time PCR of various immune cell markers of whole spleens from LIPA WT and KO littermates after cold challenge. WT: wildtype male littermates; KO: LIPA deficient male littermates. **n = 5**

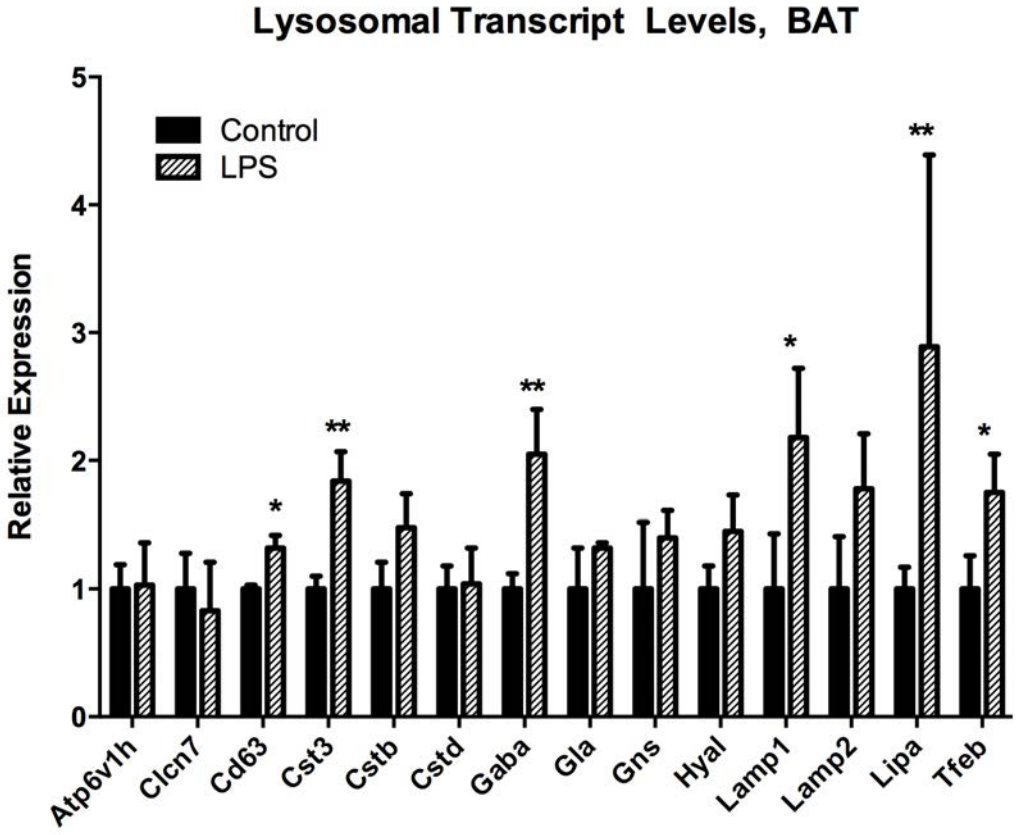
Figure 2.40



Increased lysosomal associated gene transcription in *Drosophila* larvae after mild cold exposure. Quantitative real-time PCR of whole *Drosophila* larvae after two hours in 18 C.

n = 5

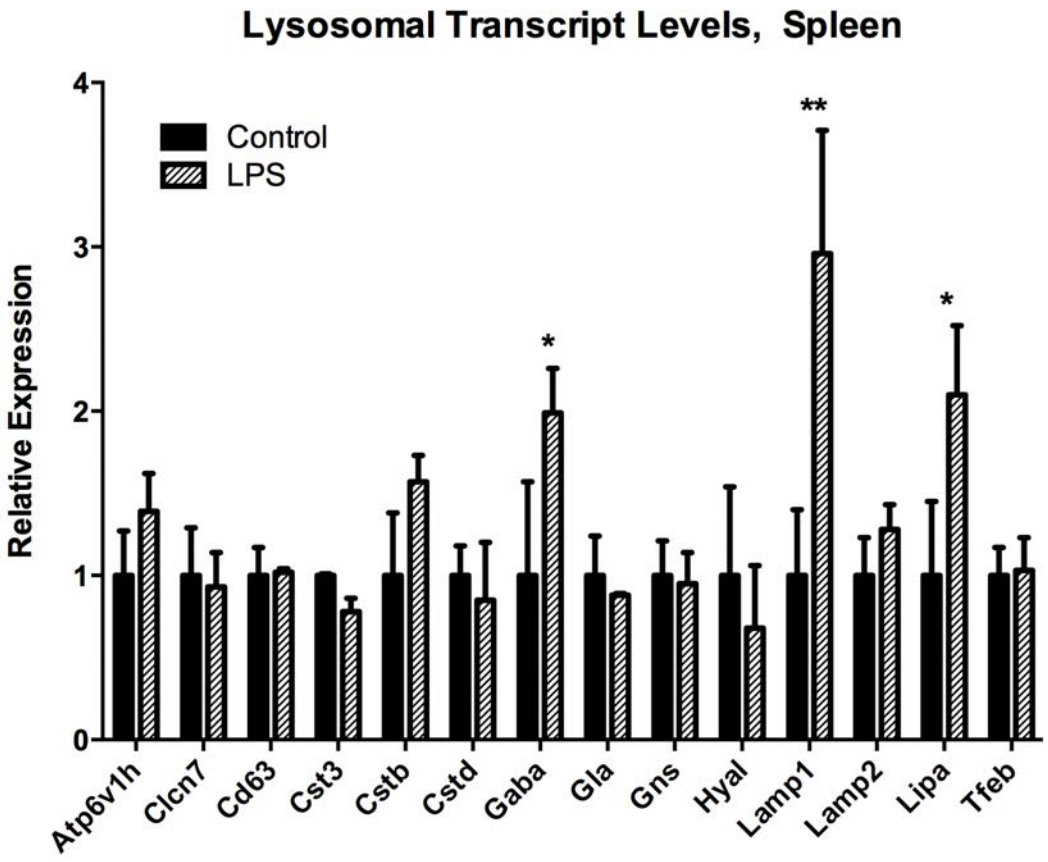
Figure 2.41



LPS injections increase transcription of lysosomal associated genes in BAT. qRT-PCR results of whole BAT depots after significant body temperature increases post LPS injection.

n = 5

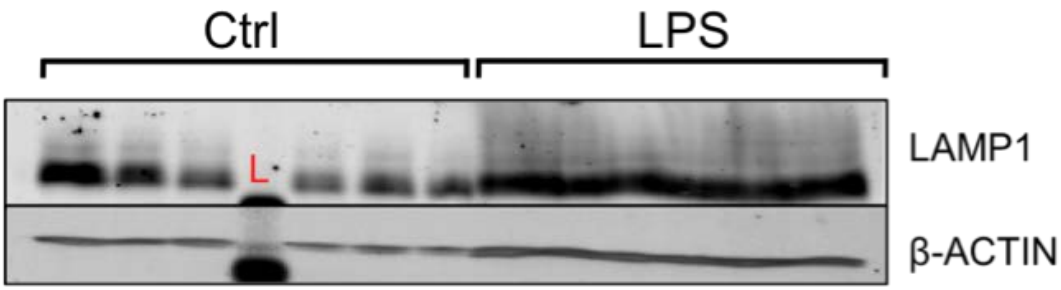
Figure 2.42



LPS injections increase transcription of lysosomal associated genes in spleen. qRT-PCR results of whole spleens after significant body temperature increases post LPS injection.

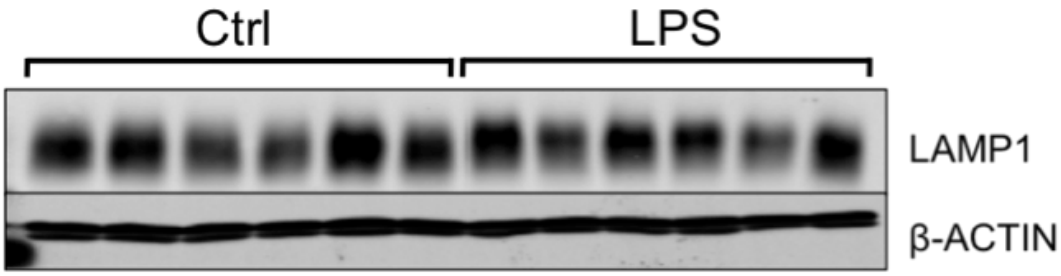
n = 5

Figure 2.43



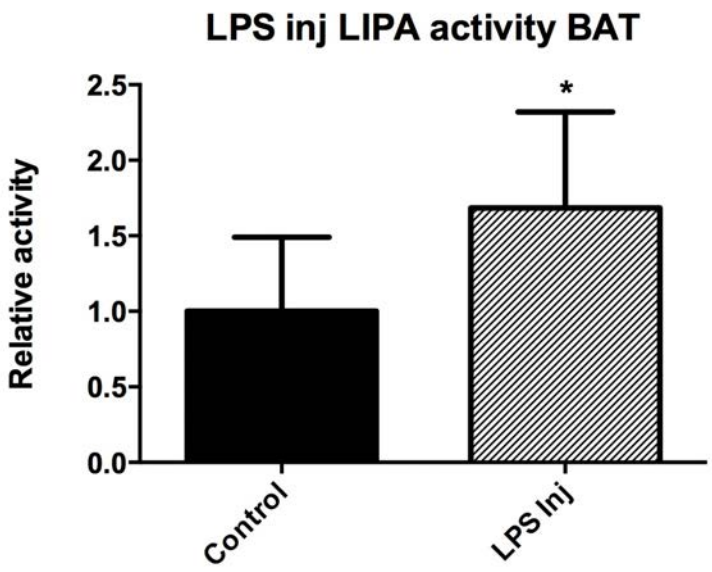
BAT LAMP1 protein expression after LPS injection. LAMP1 immunoblot from whole BAT depots after LPS injection in wildtype male mice. RT: room temperature exposed mice; CC: cold challenged male littermates; L: ladder.

Figure 2.44



Spleen LAMP1 protein expression after LPS injection. LAMP1 immunoblot from whole spleen depots after cold challenge and LPS injection in wildtype male mice. RT: room temperature exposed mice; CC: cold challenged male littermates.

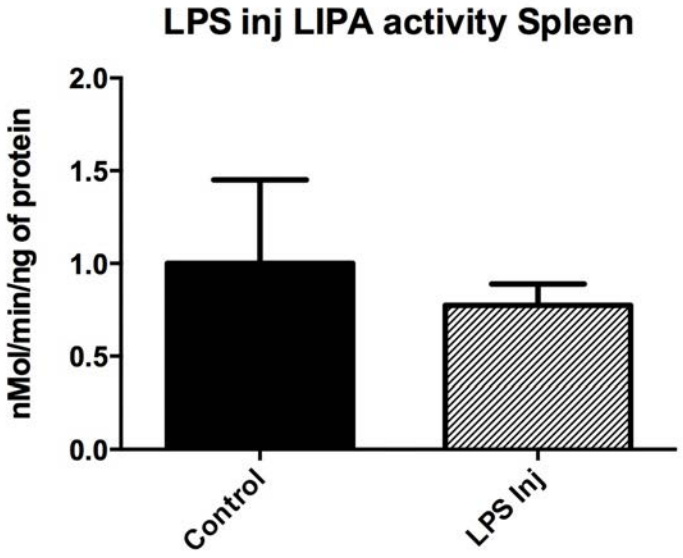
Figure 2.45



LIPA activity assay increased in BAT after LPS injections. Wildtype FVB/J mice were injected with LPS. Isolated whole BAT revealed an increase in LIPA activity, as measured by the 4-MUO assay.

n = 5

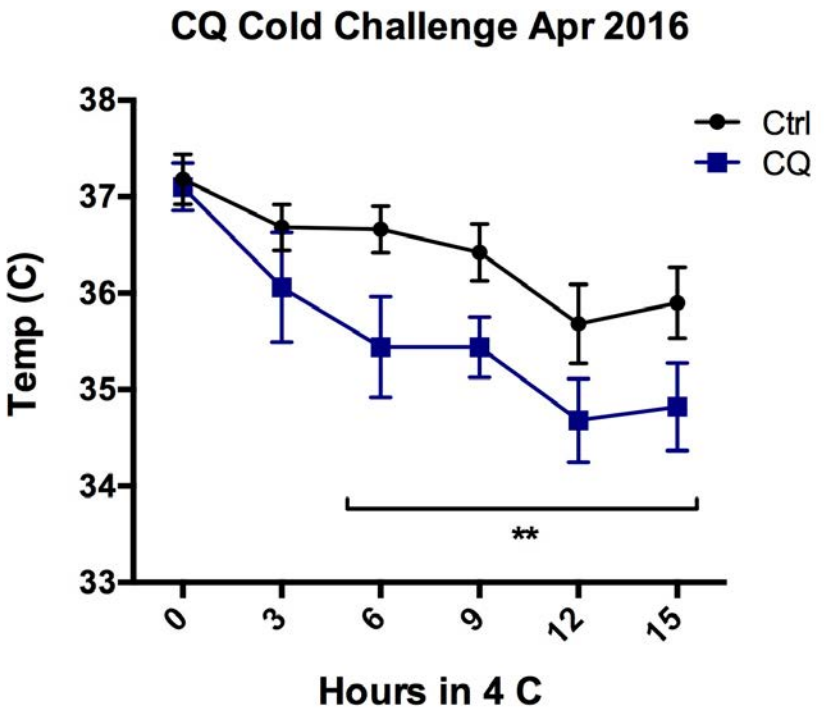
Figure 2.46



LIPA activity does not change in spleen after LPS injections. Wildtype FVB/J mice were injected with LPS. Isolated whole spleen revealed no changes in LIPA activity, as measured by the 4-MUO assay.

n = 5

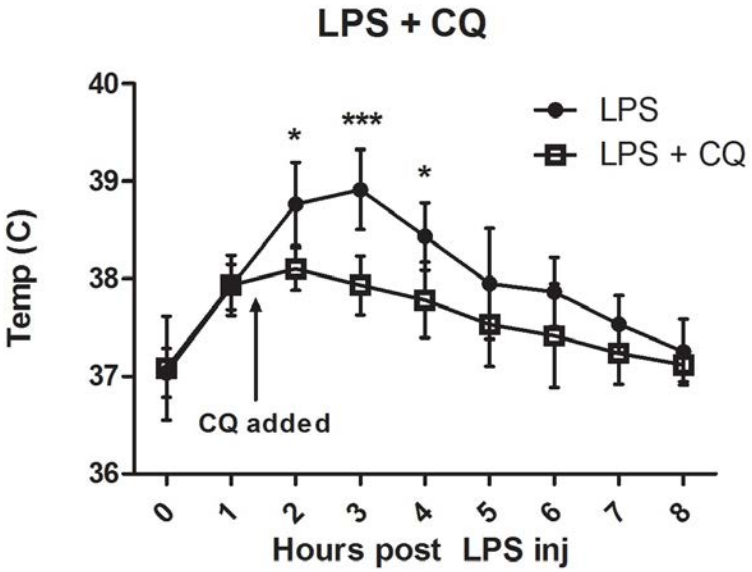
Figure 2.47



Chloroquine injected mice are cold sensitive.
Temperature data of either saline or chloroquine injected mice in cold challenge. Ctrl: saline injected; CQ: chloroquine injected.

n = 5

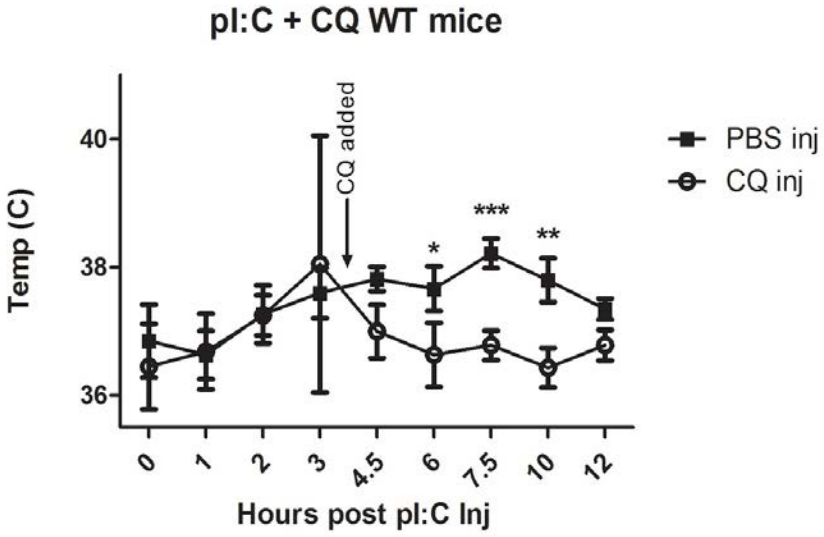
Figure 2.48



Chloroquine ablates febrile response induced by LPS. Wildtype FVB mice are injected with LPS and temperatures were monitored almost every hour. CQ was injected at 1.5 hours. After injection of CQ, temperatures were monitored every hour for eight hours.

n = 5

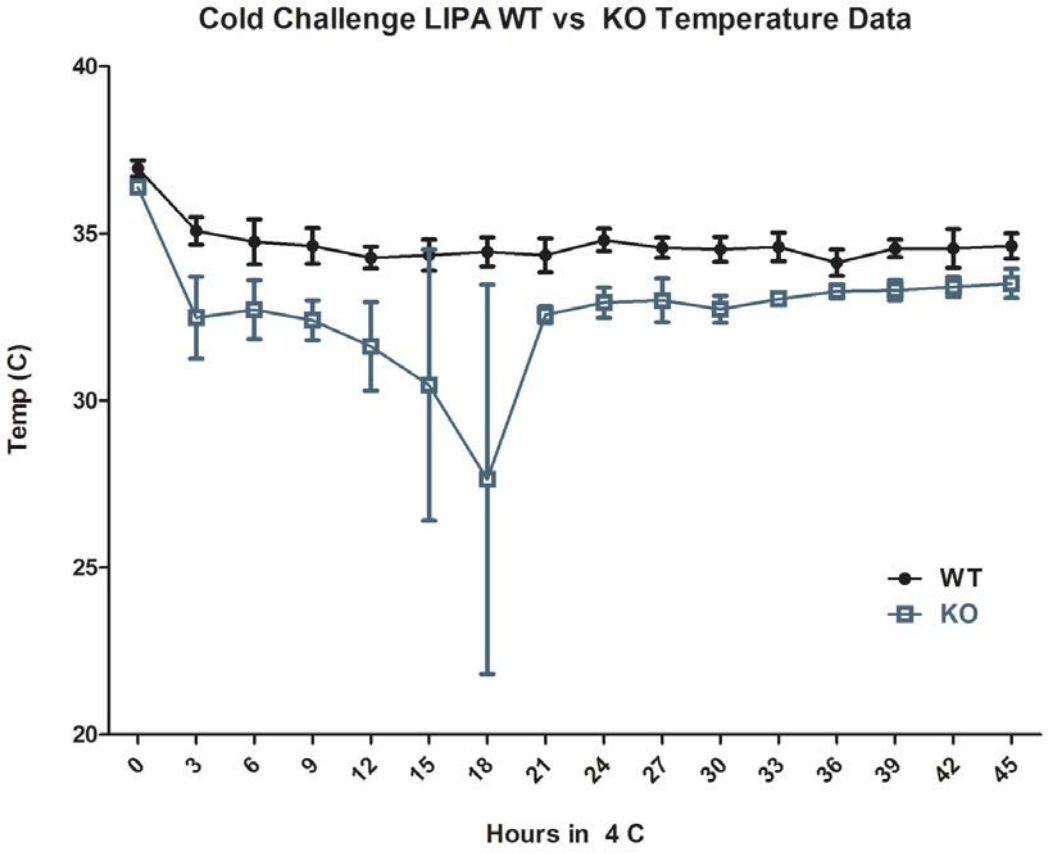
Figure 2.49



Chloroquine ablates febrile response induced by poly(I:C). Wildtype FVB mice are injected with poly(I:C) and temperatures were monitored almost every hour for three hours before CQ is injected. After injection of CQ, temperatures were monitored every 1-2 hours for a total of 12 hours.

n = 5

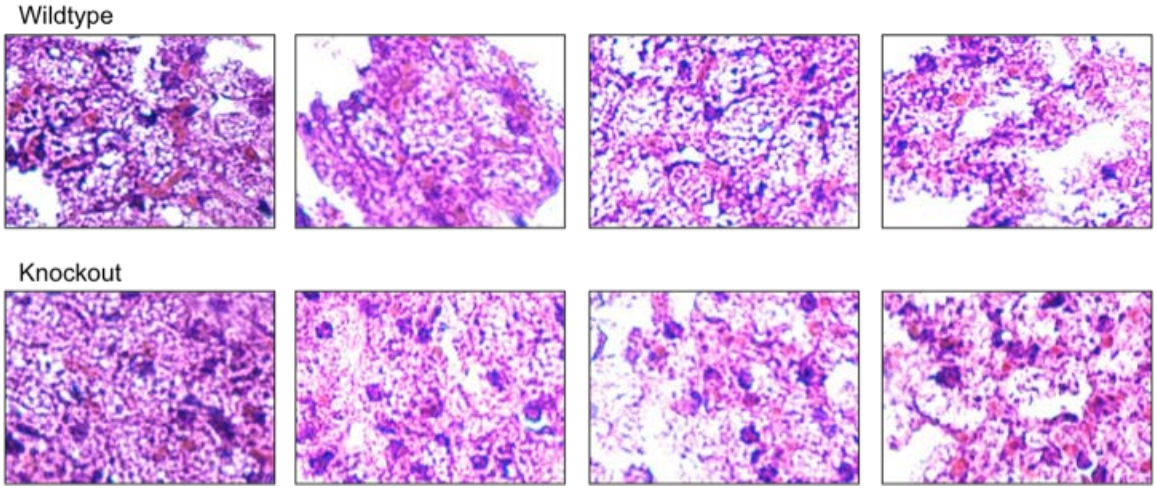
Figure 2.50



Lysosomal acid lipase deficient mice are cold sensitive. Temperature data of *Ad libitum* fed wildtype and LIPA deficient mice.

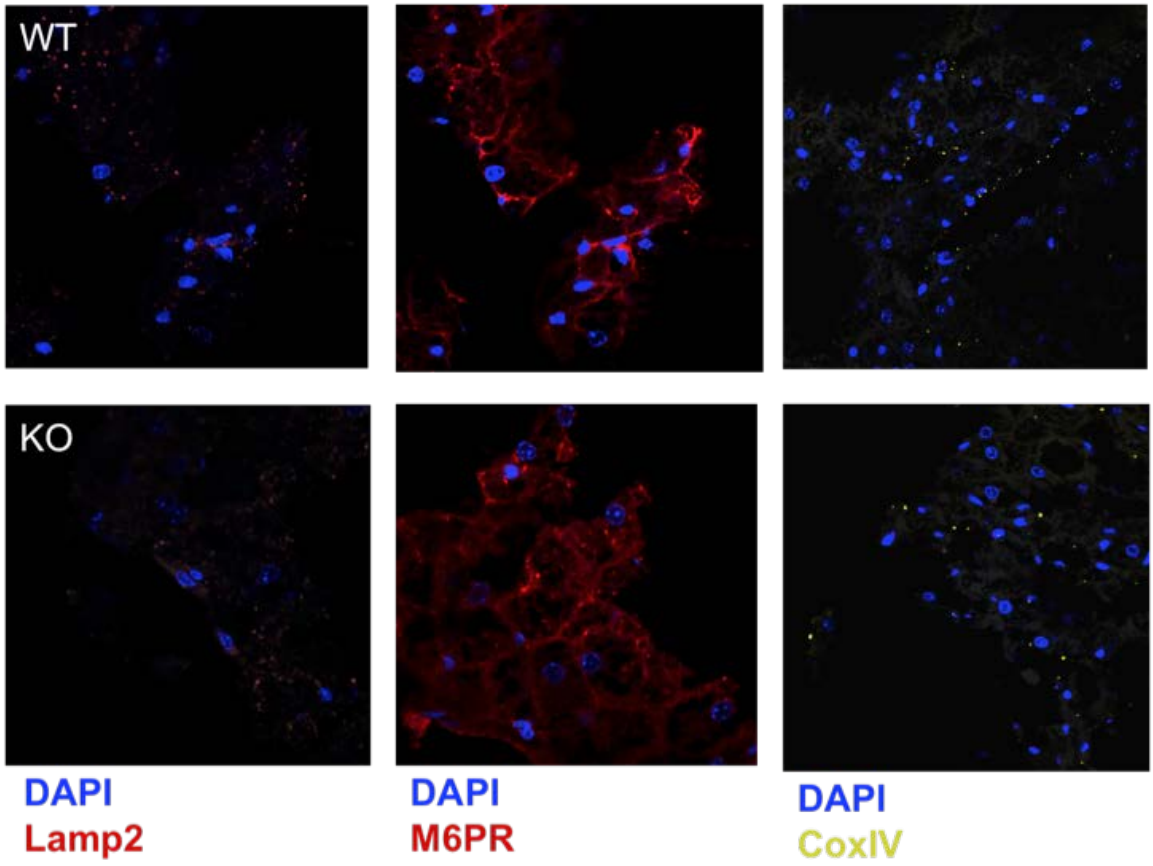
n = 6

Figure 2.51



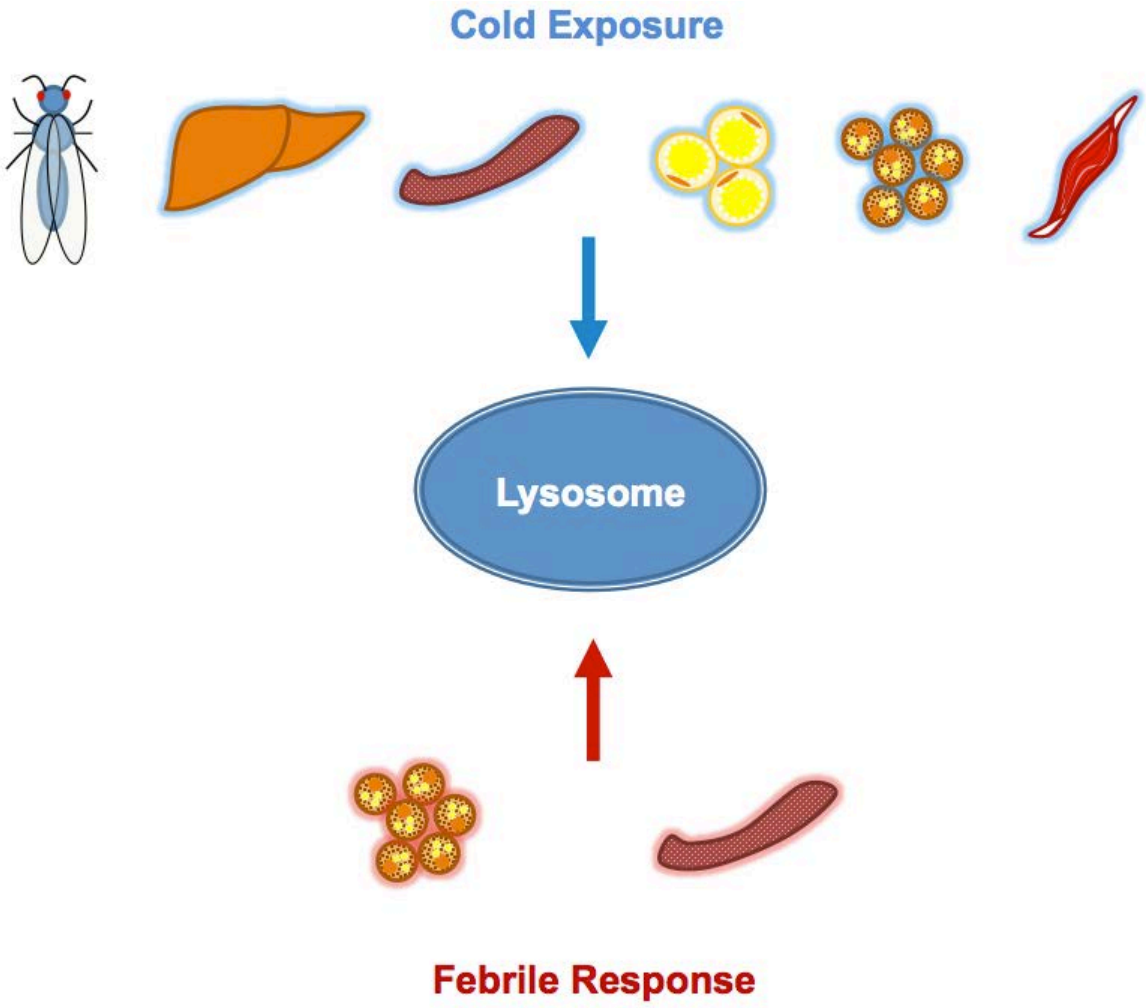
BAT histology of wildtype and LIPA deficient cold challenged mice. H&E staining of BAT sections after cold challenge in wildtype and LIPA deficient male mice. RT: room temperature exposed mice; CC: cold challenged male littermates. N = 4

Figure 2.52



Immunofluorescence of wildtype and LIPA deficient cold challenged mice. Immunofluorescent staining of BAT sections after cold challenge in wildtype and LIPA deficient male mice of LAMP2, M6PR, and COXIV. WT: wildtype male littermates; KO: LIPA deficient male littermates. N = 4.

Figure 2.53



Summary model. *Drosophila*, liver, spleen, white adipose tissue, brown adipose tissue, and muscle all show evidence of association with lysosomes during cold challenge. Both brown adipose tissue and the spleen are additionally associated with lysosomes during fever induction. Causative experiments disrupting lysosome function either pharmacologically or genetically indicate that intact lysosome activity is necessary for homeothermy.

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CHAPTER THREE

LYSOSOMES ARE SIGNALING REGULATORS OF NONSHIVERING THERMOGENESIS

Introduction

The previous chapter established that thermogenic stimuli induce lysosome activity and impairment of lysosomal function reduces body temperature in animal challenged with cold and febrile stimuli. However, it is unclear how impairment of lysosome function reduces thermoregulation in mice. We propose three possible mechanisms: 1) Signaling function of lysosomes regulate known thermogenic signals and uncoupled oxidation in BAT; 2) Lysosomes provide substrates for uncoupled oxidation in BAT; 3) Lysosomes function as thermogenic organelles, generating heat directly.

In this chapter, we will explore the effects of altered lysosomal function on canonical pathways that regulate BAT thermogenesis. Previous studies have shown that the master transcriptional regulator of lysosomes, TFEB, is also involved in the regulation of lipid catabolism in part through PGC1α (Settembre, De Cegli, et al. 2013), itself a transcriptional regulator of mitochondrial function. In this chapter, we will explore how cold challenges affect the lysosomal signaling machinery and whether impairments in lysosomal function alter canonical thermogenic pathways. If lysosomal-dependent signal is critical for these pathways, we predict a cold challenge will induce TFEB activation in turn causing PGC1α binding to the promoter of *Ucp1*, allowing for the critical thermogenic gene to be transcribed in response to cold challenge.

Finally, this chapter will explore other questions regarding the molecular signaling mechanisms involved in our lysosome phenotype. If downstream pathways such as NST and lysosomal lipid catabolism transcription are altered, then upstream regulation and stimulation of NST and lysosomal action may be altered in our LIPA deficient model. Examination of these activators, such as β3-AR initiation and fasting will be studied.

Proposed Hypothesis and Model

Lysosomes regulate thermogenic responses by brown adipocytes through the indirect control of nonshivering thermogenesis associated genes.

Methods and Materials

Animals and Animal Care.

FVB/NJ mice were obtained from the Jackson Laboratory at 6-7 weeks of age, or from the laboratory stock of lysosomal acid lipase (LIPA) mice, originally generously provided by Hong Du, PhD of Indiana University. LIPA knockout mice are missing exon 5 of the LIPA gene, resulting in a dysfunctional LIPA transcript. Experiments were performed on littermates. Mice were housed in ventilated Plexiglas cages within specific pathogen-free barrier facility maintained in a 12-hour light/12-hour dark cycle. Regular water and chow diet were provided *ad libitum*. LIPA deficient mice were genotyped using polymerase chain reaction screening of tail DNA. All procedures were approved by the Columbia University International Animal Care and Use Committee.

Quantitative Real-time Polymerase Chain Reaction.

Tissue samples were immediately frozen by liquid nitrogen from euthanized mice at the termination of experiments. RNA was extracted from frozen tissue samples using an acid-phenol reagent according to manufacturer's instructions (RNA-solv reagent, Omega Bio-tek). RNA was further purified by silica-membrane method from the manufacturer (E.Z.N.A. Total RNA Kit II, Omega Bio-tek) and then used as template for cDNA synthesis (qScript cDNA SuperMix, Quanta Biosciences). Quantitative real time PCR (qRT), was performed using white 384 well plates in the Roche LightCycler 480 machine. Analysis was done by normalizing expression to *Rps3* and expressed in relative units using the DDCT-2 method, taking account of each primer efficiency calculated per in-run standard curve. Primer sequences are listed in Table 1.

Immunoblots.

Tissue samples were homogenized by sterile stainless steel beads with QIAGEN's TissueLyser LT in tissue lysis buffer (Tissue extraction reagent I, Thermo Fisher) treated with protease and phosphatase inhibitors (Protease inhibitor cocktail I, phosphatase inhibitor cocktail II and III, SIgma Aldrich). Protein concentrations were determined by standard BCA assay (Bio-Rad). Crude protein extracts were centrifuged to separate the lipid fraction from the aqueous layer three times to purify protein. Purified protein extracts were gel electrophoresed using 10% SDS/PAGE gels, transferred to PVDF .2 um membranes (Millipore), and immunostained according to standard methods. Antibodies used: UCP1 (Abcam), LAMP1 (eBiosciences), LAMP2 (Abcam), PGC1 (Santa Cruz Antibodies), PPARy (Cell Signaling Technologies), phospho-p38MAPK (Cell Signaling Technologies), p38MAPK (Cell Signaling Technologies), b-actin (Sigma-Aldrich), a-tubulin (Santa Cruz Antibodies). Blots were visualized using LI-COR fluorescent secondary antibodies and a Li-COR imager.

Protein densitometry.

Protein densitometry was calculated using ImageJ. All protein are normalized to either beta-actin or alpha-tubulin for whole cell or cytoplasmic fractions. TATA-binding protein (TBP) (Abcam) was used as the control protein for nuclear fractions.

Lysosomal acid lipase activity assay.

LIPA activity was determined using a 4-methylumbelliferyl oleate assay with 4-methyumbelliferone serving as the standard curve. 4-MUO was dissolved in hexane at 100 mg/ml and diluted 1 to 250 in 4% Triton X-100. Tissue extracts were prepared in the same way as western blot samples (see *Immunoblots*). 25 ul of diluted tissue extracts, 50 ul of diluted 4-MUO substrate, and 125 ul of assay

buffer (0.2 M NaOAc and 0.01% Tween 80, pH 5) were incubated at 37flC for 30 minutes. To stop the reaction, 100 ul of 0.75 M Tris, pH 8.0 was added to each sample. Fluorescence was detected using (**look up machine**) at excitation 360 nm and emission 460 nm. Relative fluorescence units from each sample were then compared to that of the standard curve and normalized to wildtype controls.

Histology, Immunohistochemistry, and Immunofluorescence.

Histology was performed by Columbia University Medical Center Russ Berrie's Pathology Core. Sections were sliced at 5um and paraffin-embedded. For IHC staining, adipose tissues were fixed with Z-FIX for 48 hours and embedded in paraffin. Tissue sections (5mm thick) were deparaffinized, rehydrated, and followed by antigen retrieval step before being stained with ABC/DAB (Vectastain) kit followed by counterstain. Antibodies used for IHCs: UCP1 (Abcam). For IF staining, adipose tissues were fixed in 4% PFA for 24 hours, followed by 10% sucrose and embedded in OCT before freezing. Tissue sections (5mm thick) followed the standard IF protocol described by AbCam. IF slides were mounted using Invitrogen's ProLong Gold antifade reagent with DAPI (Invitrogen). Antibodies used: LAMP1 (eBiosciences), LAMP2 (Abcam), PLIN1 (Abcam), and M6PR (Abcam).

Chloroquine injections.

Chloroquine (Chloroquine diphosphate salt, Sigma-Aldrich) was dissolved in sterile water at a concentration of 100 nM. 100 uL of 100 nM chloroquine was injected subcutaneously in the interscapular region of mice. Littermate controls were injected with 100 uL of sterile water. Mice were rested for two hours before subsequent procedures were performed.

Cold challenge.

Mice were housed individually for up to 72 hours in 4-8flC. Cold challenge experiments for this chapter allowed *ad libitum* access to regular chow diet and water. Mice followed the same 12 hr day/12 hr night cycle of the Columbia ICM specific pathogen free housing.

In vivo temperature monitoring.

To measure the core body temperature of mice during cold challenge, mice were placed on wire lids and tails lifted to expose the anal region. Once exposed, a rectal temperature probe was inserted to determine internal body temperature. Temperatures were recorded after initial equilibration (approximately 4-7 seconds).

Food intake.

Food intake was measured by recording weights of food hoppers with food prior to cold challenge and post cold challenge.

Statistics.

Significance calculations of comparisons between two groups were determined by student's t-test. Significance calculations of multiple group comparisons were determined first by ANOVA and then Bonferroni correction. Key for all significance indications: * = p-value ≤ 0.05; ** = p-value ≤ 0.01; *** = p-value ≤ 0.001.

Figures represent one experiment out of four. Data are not combined to produce the figures or statistics. Error bars represent the standard deviation.

Results

Chloroquine injections in cold challenge mice increase thermogenic signaling

We found that chloroquine (CQ) impairs thermogenic response to a cold or febrile challenge (Figures 2.47-2.49). A known effect of inhibiting lysosome acidification and function is a compensatory upregulation of lysosomal transcripts and protein production. Indeed, we found that chloroquine treatment induced lysosomal transcripts and LIPA activity in BAT (Fig 3.1-3.2), indicating that our injection of CQ into BAT had inhibited lysosome function. We hypothesized that impaired lysosome function would reduce thermogenesis via the canonical uncoupled program in BAT. This mechanism would predict CQ induced reduction in expression of NST-associated genes typically upregulated during a cold challenge. Surprisingly, however, we found that the NST associated genes, *Cidea*, *Pgc1α*, *Pparα*, and *Ucp1* (Figure 3.1) were upregulated in BAT. SUBQ also saw an increase in two NST associated genes, *Cidea* and *Ucp1* (Figure 3.2). These data suggest an attempted compensatory increase in canonical NST thermogenesis in BAT and that CQ-induced cold sensitivity is not a result of impaired thermogenic signaling.

Thermogenic transcriptional programs in brown and beige adipocytes of LIPA KO mice.

Given the thermogenic impairment of LIPA deficient mice, we were curious to see whether canonical NST signaling was affected by long-term impairment of lysosomal function. *Lipa-/-* and control littermate mice were exposed to 4-8 C for 72 hours. We assessed the expression of genes implicated in regulating uncoupled mitochondrial oxidation. Unlike CQ treatment, LIPA deficiency did reduce the

BAT expression of several transcripts implicated in canonical NST, including *Pgc1α* and *Ucp1* (Figure 3.6). These gene expression data were corroborated by immunoblots of PGC1, PPARɣ, and UCP1 (Figure 3.7 and 3.8). These observations suggest that lack of LIPA does impair NST signaling in brown adipocytes.

Our pharmacologic and genetic data provide somewhat conflicting evidence for the role of lysosomes in the canonical NST UCP1 transcriptional program. The life long deficiency in LIPA reduced the expression of the NST program while short-term inhibition of lysosomes did not. In mice, SUBQ fat contains both typical white adipocytes as well as a population of thermogenic cells, beige adipocytes (Giralt and Villarroya 2013; Harms and Seale 2013). When a mouse is cold challenged, adrenergic and local signal active the classic UCP1 thermogenic program in beige adipocytes. Indeed, the induction of UCP1 is typically an order of magnitude greater in subcutaneous compared to brown adipose tissue. If LIPA deficiency were incompatible generally with the canonical NST signaling, we would expect a reduction in the expression of the genes in this pathway. We assessed the canonical transcriptional program in SUBQ following a cold challenge of 4-8 C for 72 hours.

Unlike BAT, lack of LIPA in SUBQ fat had higher expression of genes that form the canonical NST program (Fig 3.9). These included augmented levels of *Pgc1α* and *Ucp1* expression. Further validating these data, we found heightened PGC1, PPARɣ, and UCP1 protein levels (Fig 3.10, 3.11). Finally, immunohistochemistry of sectioned SUBQ tissue display more UCP1 staining in LIPA KO mice cold challenged mice compared to their wildtype control counterparts (Figure 3.12).

These data, contrary to what was observed in the BAT of LIPA KO mice, indicate that NST is not only intact, but also enhanced in the SUBQ of LIPA KO mice.

Upstream regulators of nonshivering thermogenesis signaling is enhanced in both brown and subcutaneous adipose tissue

In various models in which we inhibit lysosomal function, we observed apparent compensatory effects, with hyperactivation of lysosomal programs in BAT (CQ treated animals) and SUBQ (CQ treated & *Lipa*-/- animals). We searched for evidence that signaling upstream of the canonical NST program is upregulated.

NST initiation in the brown adipocyte begins with the activation of the β3-adrenergic receptor (β3AR). Following its activation, a signaling cascade begins, eventually resulting in the phosphorylation of p38-MAPK, the upstream regulator that is necessary for the actions of PGC1α as well as a number of additional proteins and transcription factors crucial in the induction of thermogenesis (Tseng et al. 2010). As p38-MAPK is one of the last cytoplasmic proteins in the pathway before transcriptional regulation of NST begins, we measured phosphorylation of p38-MAPK as an indicator of an intact central regulatory system. We found an increase in phospho-p38-MAPK levels in our LIPA deficient mouse models compared to their wildtype littermates (Fig 3.13 and 3.14). Our data argue that the defect in BAT thermogenic signaling is not due to decreased upstream signal; rather, there is increased upstream signaling that is occurring as an adaptive response to reduced thermogenesis.

Master regulators of lysosomal biogenesis and lipid catabolism are not activated during a cold challenge in wildtype mice.

As seen previously, a cold challenge activates a lysosome transcriptional program in select tissues of mice. Lysosomal regulation is primarily dependent on TFEB and the related proteins, TFE3 and MITF (Mony, Benjamin, and O'Rourke 2016; Settembre, Fraldi, et al. 2013; Ploper and De Robertis 2015; Sardiello et al. 2009). These transcription factors interact with the lysosome via the LYNUS machinery. Their sequestration by mTORC1 prevents translocation to the nucleus and transcription of the lysosomal program. Initial cold challenge experiments indicate an increase in the expression of *Tfeb* in cold challenged wildtype mice and other lysosomal genes controlled by TFEB (Fig 3.15).

To determine whether a cold challenge activates the lysosome transcriptional program via the LYNUS machinery and TFEB translocation, we measured protein localization and how it was affected by a cold challenge. BAT from room temperature and cold challenged mice were extracted from wildtype and LIPA knockout mice. Tissues were then processed to two fractions: cytoplasmic and nucleic. We quantified TFEB protein in the nuclear and cytosol using standard immunoblotting techniques.

Our study of wildtype mice showed that the lysosomal transcriptional program was not activated by translocation of TFEB to the nucleus. (Figure 3.16 and 3.17). The lack of translocation differences between room temperature and cold challenged BAT samples suggest that TFEB, and other currently known lysosomal and lipid catabolism transcription factors cannot explain either the

lysosomal transcriptional program or the NST signaling regulation in wildtype mice.

Master regulators of lysosomal biogenesis and lipid catabolism are partially impaired in the brown adipose tissue of LIPA KO mice. Our gene expression studies of BAT from LIPA-deficient mice reveal lysosomal biogenesis and lipid catabolism genes are reduced in the BAT of LIPA KO mice (Figure 3.18). We therefore examined TFEB, TFE3, MITF, and MAX protein data in subcellular fractions of cold challenged versus room temperature LIPA deficient BAT.

We found little expression of TFE3 and MITF in BAT, again suggesting that these two proteins are unlikely to play critical role in regulating lysosomal biogenesis. However, our TFEB western blots suggest that translocation may be blunted in the knockout mice (Figure 3.19 and 3.20). TFEB is both a transcriptional regulator of lysosomal function as well as lipid catabolism genes, in particular PGC1α. As a result, our LIPA deficient model may have defects in lipid catabolism and lysosome function due problems in TFEB translocation.

β-3 adrenergic receptor stimulation requires fasting to activate a lysosomal program.

Cold challenge activates several pathways and systems in homeotherms. The best studied and one required for thermogenesis is adrenergic stimulation of BAT. Adipocytes, including brown adipocytes, express the β3-adrenergic receptor (β3AR) which is sufficient to activate the canonical thermogenic program of

BAT. Therefore, to determine whether β3AR stimulation alone is sufficient to initiate a lysosomal response, we treated mice with the β3-adrenergic receptor agonist, CL-316,243 (CL). Surprisingly, CL treatment alone was insufficient to activate Both mRNA and protein data show that β3AR stimulation alone fail to induce a lysosomal phenotype in the BAT and only modestly increased *Ucp1* expression. However, when fasting is combined with β3AR stimulation, we saw a significant induction of a lysosomal phenotype and expression of *Ucp1* (Fig 3.21). LIPA activity of BAT was similarly regulated, ie only the combination of fasting and β3AR stimulation induced LIPA activity (Fig 3.22). These data suggest that cold challenge mimics both a negative energy balance and β3AR stimulated states.

Proposed summary model

The data presented in this chapter suggest that pharmacologic inhibition of lysosomal function in a way that reduces thermogenesis but does not impair canonical NST signaling. The effects of genetic inhibition of lysosome function are more complex. LIPA deficiency does reduce the UCP1 dependent transcriptional response to a cold challenge modestly (~ 50%) in BAT but not in SUBQ fat. Together these data argue that lysosomal function is not absolutely required for a canonical thermogenic response. However, it also suggests that LIPA activity modulates at least in the thermogenic response in BAT.

Our data also suggest that the signaling upstream of the classical NST response is intact when lysosomal function is impaired. Specifically, the canonical β3-

adrenergic receptor activated pathway is intact up to p38-MAPK phosphorylation, which is upregulated when the contribution of lysosomal thermogenesis is reduced in LIPA-deficient or CQ treated mice. Therefore, this observation indicates not only a functional central response, but also a largely intact intracellular initial signaling response in the brown/beige adipocyte.

The coordinated upregulation of lysosomal genes in response to a cold challenged lead us to hypothesize that placing mice at 4˚C would induce TFEB nuclear translocation in BAT. Unexpectedly, we did not observed the expected cold-induced nuclear translocation. These findings suggest another mechanism by which a lysosomal transcriptional program is activated.

Conclusions

In our previous chapter, we were able to demonstrate the importance of lysosomal acid lipase in the maintenance of body temperature. Those observations did not show how LIPA may be related to temperature regulation. Here, our experiments suggest that LIPA deficiency may alter thermogenic response by directly signaling to downstream effectors of the NST pathway.

The canonical NST pathway requires the expression of Ucp1 via PGC1α activation. The brown adipose tissue of cold challenged LIPA KO mice showed impairments in UCP1, PGC1α, PPARα at both RNA and protein levels. The approximate 50% reduction in expression suggest that lysosomes may be transcriptionally involved in altering NST regulation. Of course, these disruptions may be a sign of overall cellular dysphoria from LIPA dysfunction. However, two observations put these doubts to rest: first, we show in chapter two that the gross morphologies of the BAT of LIPA KO mice are normal; second, when we look further upstream in the NST pathway, we find compensatory mechanisms to account for the NST disruption. As such, our data propose that the NST impairment in our LIPA KO mice is a result of LIPA deficiency itself.

Interestingly, while NST was impaired in the BAT of our LIPA KO mice, we found the opposite to be true in the SubQ of the same individuals. Instead, the white adipose tissue, another source of some thermogenic adipocytes, increased the transcription and thusly protein levels of the same NST genes: Ucp1 and Pgc1α. Immunohistochemistry of SubQ sections showed a robust increase in UCP1 staining. Similar suggestions of differential molecular pathways governing

thermogenic adipocytes in SubQ and BAT can be found in chapter two, where lysosomes were associated with cold challenge in BAT, but not SubQ.

Disruptions in downstream NST signaling may be explained in part by either inadequate cold sensing or activation. However, when we examined the phosphorylation of the upstream protein, p38-MAPK, we found that phosphorylation was increased in the BAT of LIPA KO cold challenged mice. This suggests a compensatory mechanism for the impaired NST response, indicating that central regulation is unlikely disrupted.

As part of our hypothesized model, TFEB may exert its actions on NST signaling via transcription of Pgc1α, a classic lipid catabolism gene and a known downstream target of TFEB. However, cold challenge data of wildtype mice demonstrate that TFEB does not translocate during cold challenge. Further examination of other members of the family, TFE3 and MITF, yielded unsatisfactory answers due to limitations in the antibodies. Nonetheless, we found that these master regulators of lysosomal biogenesis and lipid catabolism are partially impaired in the brown adipose tissue of LIPA KO mice. Further, other lysosomal biogenesis genes and proteins are decreased in LIPA knockout mice during cold challenge.

Finally our data suggest that the lysosomal phenotype during cold challenge is attributed to not only adrenergic stimulation but also catabolic stimuli, suggesting a synergistic crosstalk between the two pathways.

Limitations and Concerns

Tissue specificity.

The signaling data presented in this chapter are based off of our whole body knockout of LIPA. Therefore, all our data must be interpreted in the whole body context. Nonetheless, even in the ideal situation where our studies can be conducted in an inducible, tissue specific ablation of LIPA, lysosomal enzymes can be secreted and taken up by other cell types. Bone marrow transplantation of wildtype into knockout mice and vice versa result in LIPA in nearly all tissues, rescuing the severe lipodystrophic phenotype and hepatosplenomegaly in knockout mice. In fact, the basis of enzyme replacement therapy for lysosomal storage disease patients is dependent on the uptake of lysosomal enzymes in cells. While this phenomenon is certainly fortunate for those patients, it provides an extra barrier for research studies as an inducible, temporally controlled, tissue specific knockout would likely be no different than a wildtype sibling outside of their genetics.

Cell type.

Similar to the limitation already mentioned in the previous chapter, another major drawback to this study is the lack of distinction among cell population types within the tissues. This heterogeneity may have masked some of the effects that we could have seen in brown adipose tissue.

Technical.

The discoveries of TFEB, TFE3, MITF, MAX, are relatively recent. It is unclear what their expression levels are in the various tissues in our mice, particularly as the majority of studies have been performed in the liver. While we had a functional TFEB antibody, the nonspecific bands of our immunoblots overlapped

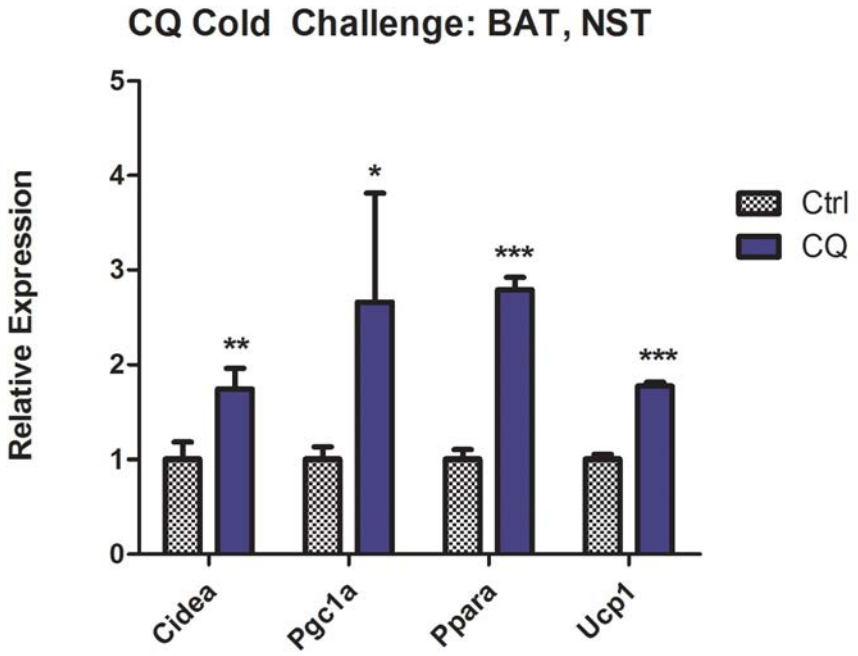
with TFE3 and MITF, making it difficult to distinguish the differences among the various transcription factors. Moreover, both TFE3 and MITF antibodies proved troublesome, and we found that interpretation of the data to be impossible.

Other transcription factors.

Given the nonspecific nature of the TFEB antibody, it is possible that other transcription factors outside of TFE3 and MITF may be in play. The understanding of the lysosomal signaling pathway is still in its nascent stages, and the characterization as well as elucidation of other potential transcription factors bridging lysosomal function with transcriptional regulation is beyond the scope of this dissertation.

Figures

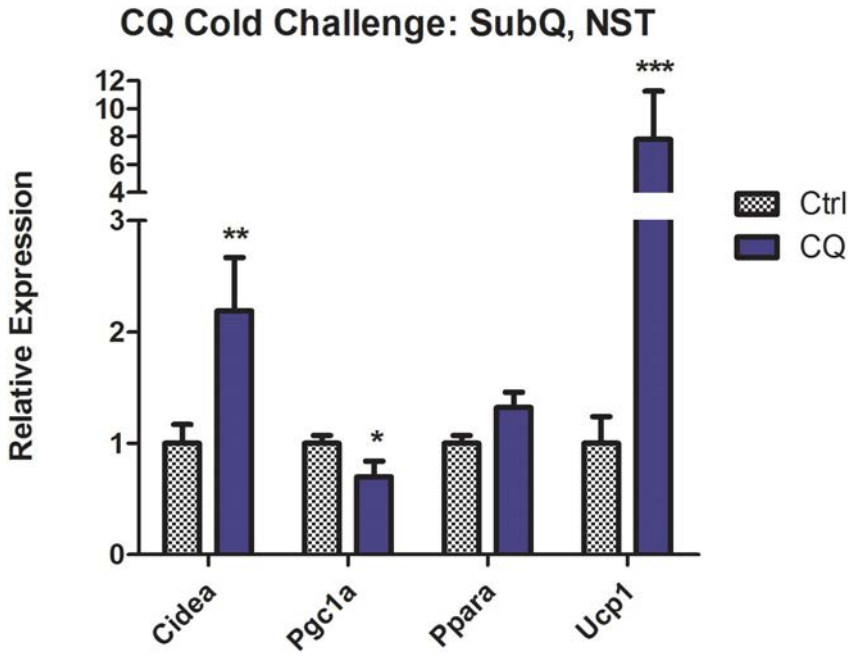
Figure 3.1



Chloroquine BAT injection increases thermogenic gene expression. Quantitative real time PCR data of four NST genes: *Cidea*, *Pgc1a*, *Ppara*, and *Ucp1*. Ctrl: saline injected, CQ: Chloroquine.

n = 6.

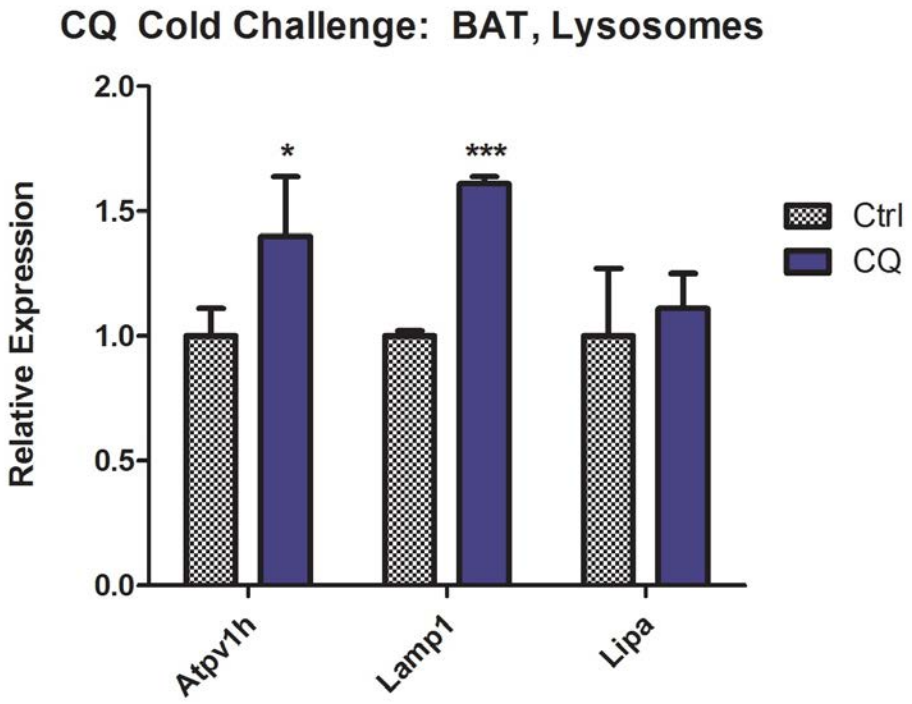
Figure 3.2



Chloroquine injection increases increases *Cidea* and *Ucp1* expression in SubQ. Quantitative real time PCR data of four NST genes: *Cidea*, *Pgc1a, Ppara*, and *Ucp1*. Ctrl: saline injected, CQ: Chloroquine.

n = 6.

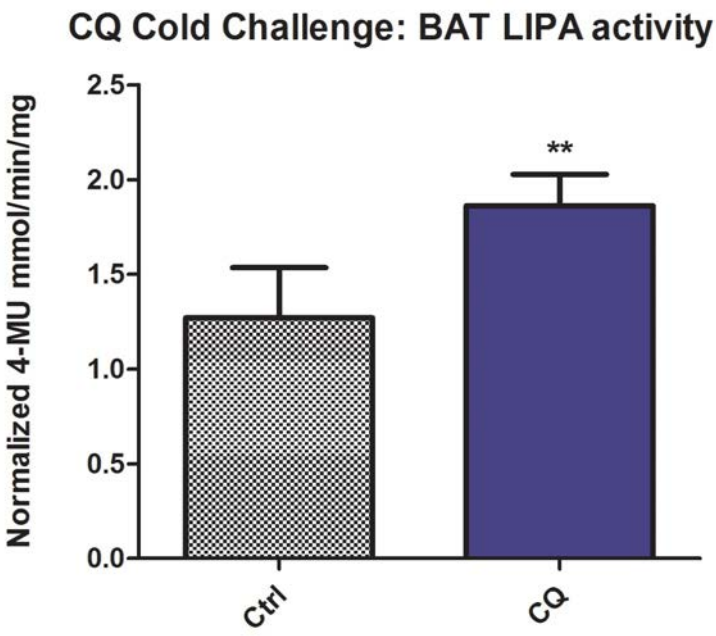
Figure 3.3



Chloroquine injection induces a compensatory lysosomal program in the BAT. Quantitative real time PCR data of three lysosomal genes: *Atpv1h, Lamp1,* and *Lipa*. Ctrl: saline injected, CQ: Chloroquine.

n = 6.

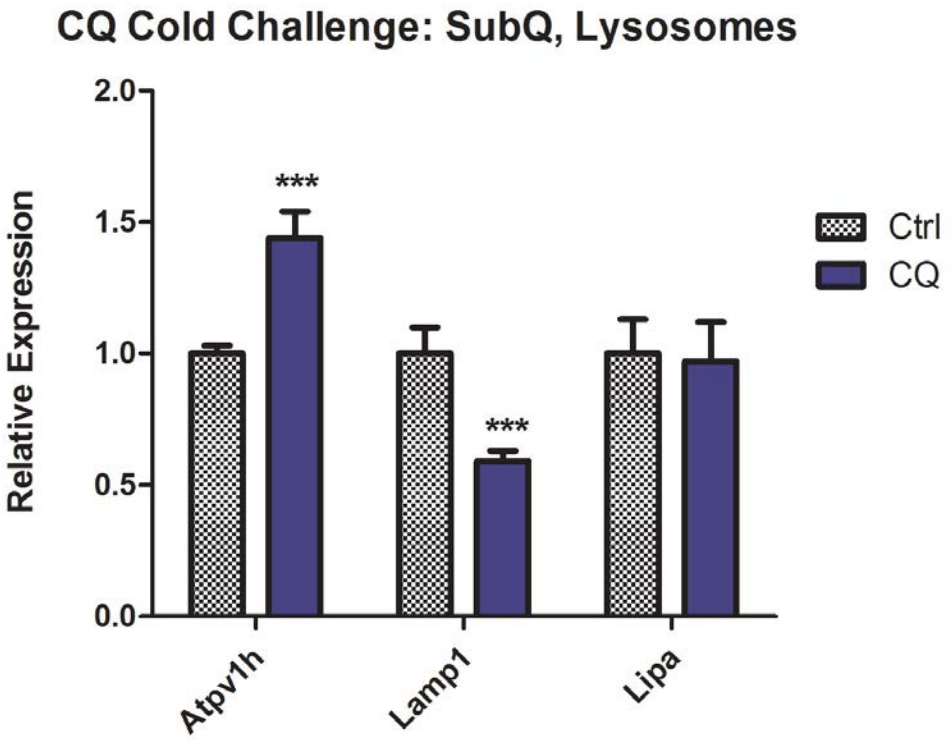
Figure 3.4



Chloroquine injection induces a modest increase in LIPA activity in the BAT. LIPA activity in whole BAT as measured by the 4-MUO assay. Ctrl: saline injected, CQ: Chloroquine.

n = 6.

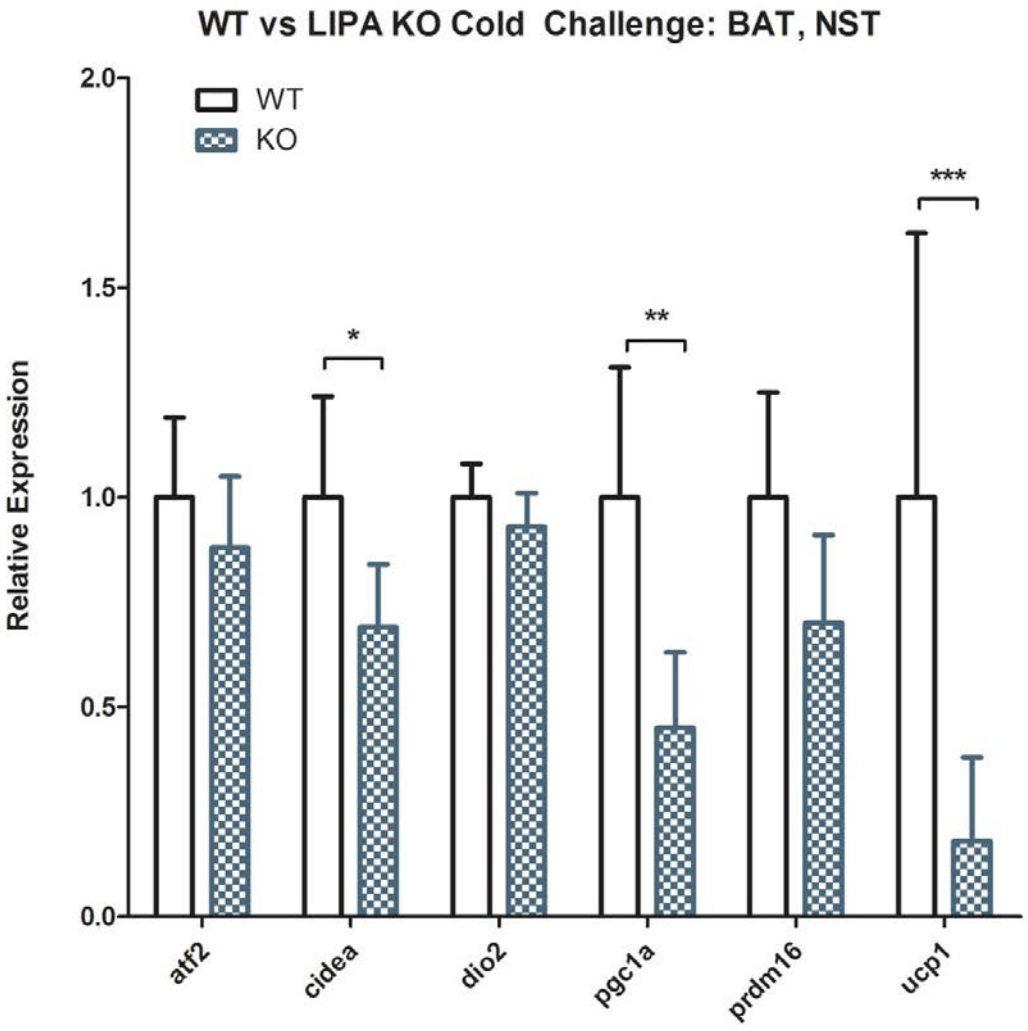
Figure 3.5



Chloroquine injection dose not induce a compensatory lysosomal program in the SubQ. Quantitative real time PCR data of three lysosomal genes: *Atpv1h, Lamp1,* and *Lipa*. Ctrl: saline injected, CQ: Chloroquine.

n = 6.

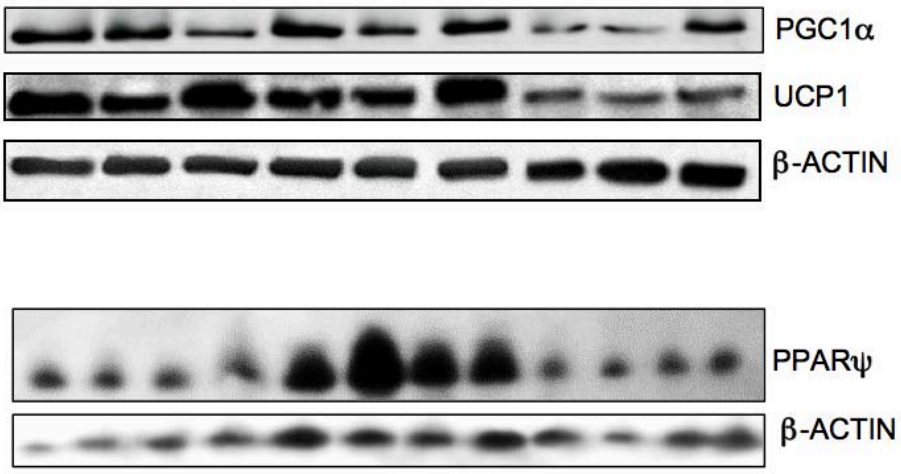
Figure 3.6



Cold challenge of LIPA KO mice results in a reduced thermogenic response in BAT. Quantitative real time PCR of six NST associated genes: *Atf2*, *Cidea*, *Dio2*, *Pgc1a*, *Prdm16*, and *Ucp1*. WT: wildtype male littermates; KO: LIPA deficient male littermates.

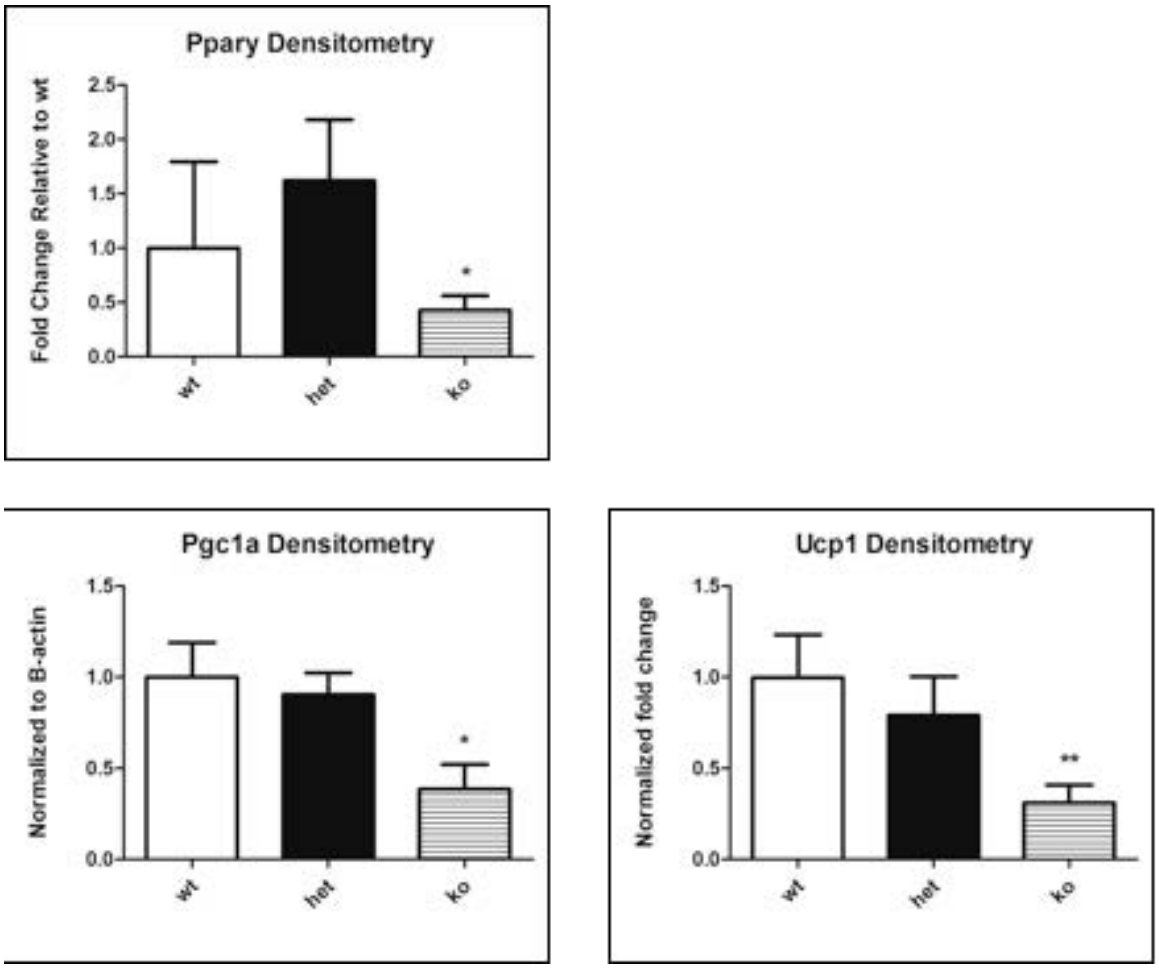
n = 5.

Figure 3.7



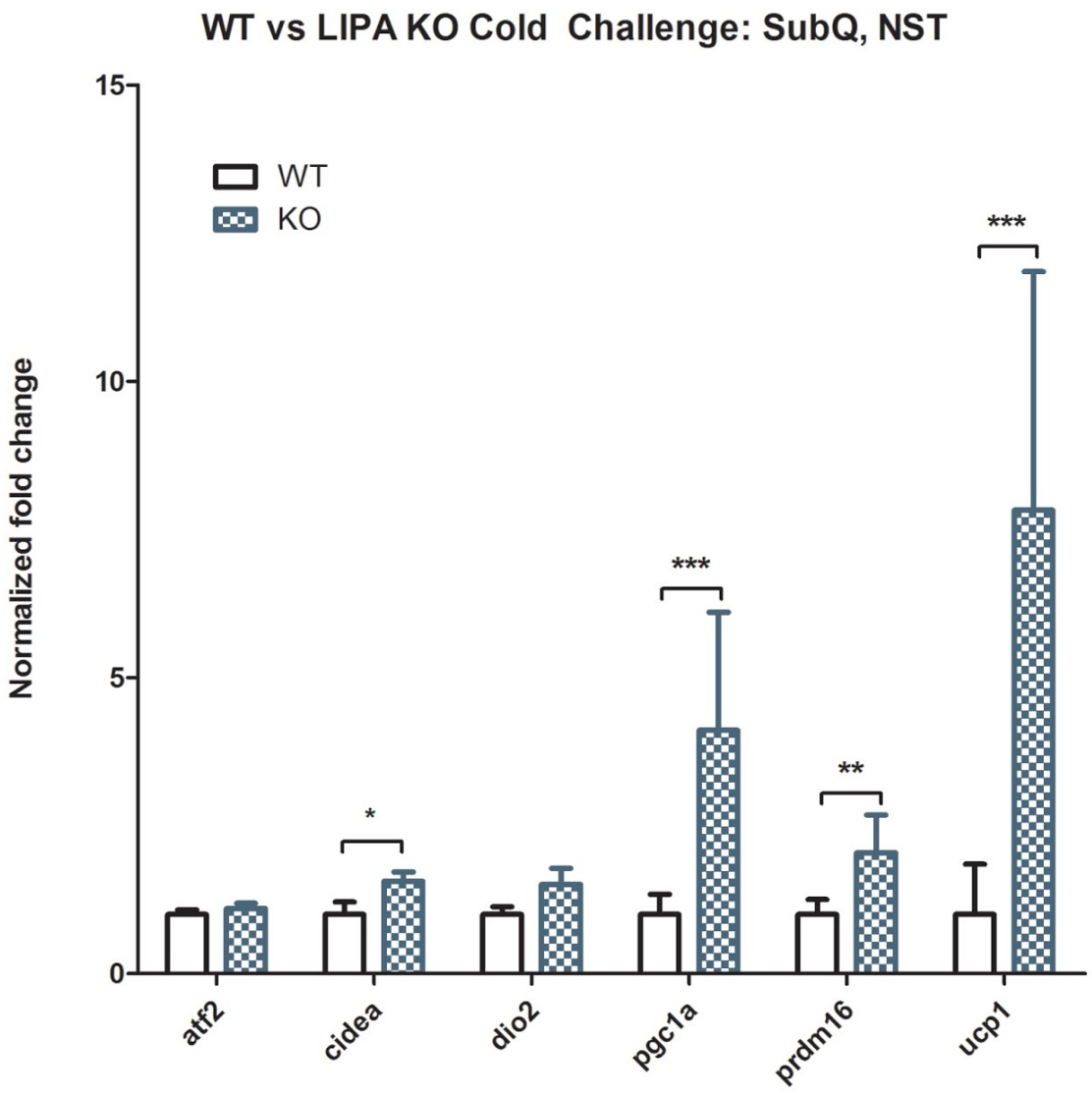
LIPA deficient mice show reduced expression of NST associated proteins. Immunoblots of three key NST-associated proteins: PGC1a, UCP1, and PPARy. WT: wildtype male littermates; HET: heterozygous male littermates; KO: LIPA deficient male littermates.

Figure 3.8



Densitometry of NST associated proteins. Densitometry of PPARy, PGC1a, and UCP1 proteins. WT: wildtype male littermates, HET: heterozygous male littermates, KO: knockout male littermates.

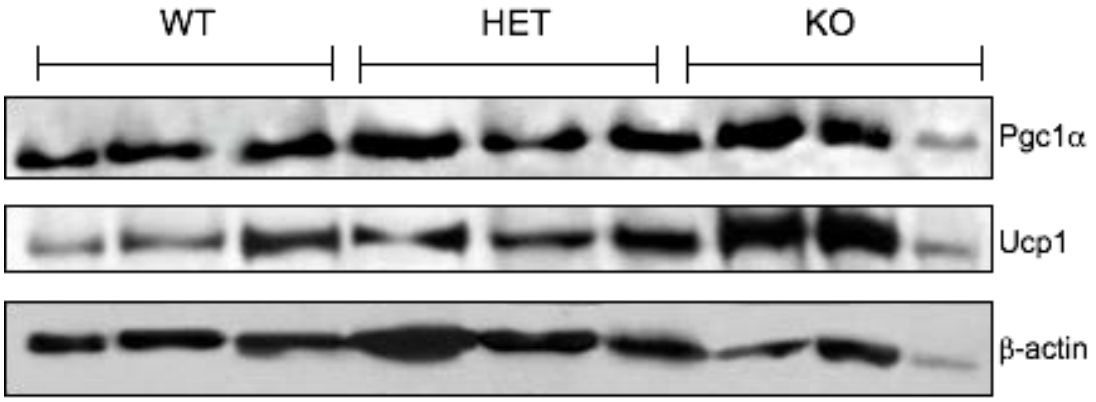
Figure 3.9



Cold challenge of LIPA KO mice results in an increased thermogenic response in SUBQ. Quantitative real time PCR of six NST associated genes: *Atf2*, *Cidea*, *Dio2*, *Pgc1a*, *Prdm16*, and *Ucp1*. WT: wildtype male littermates; KO: LIPA deficient male littermates.

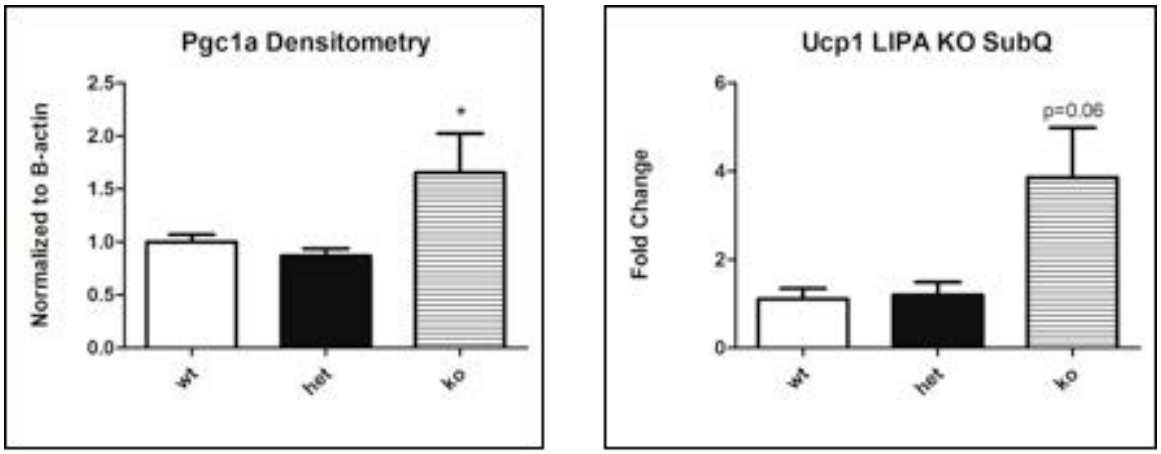
n = 5.

Figure 3.10



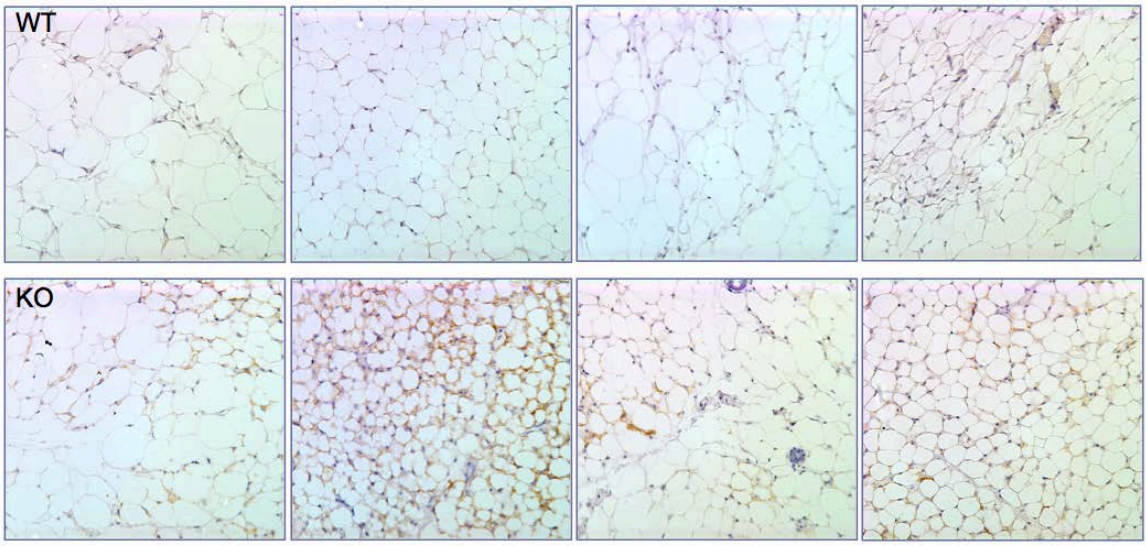
LIPA deficient mice show increased expression of NST associated proteins in BAT. Immunoblots of three key NST-associated proteins: PGC1a, UCP1, and PPARy. WT: wildtype male littermates; HET: heterozygous male littermates; KO: LIPA deficient male littermates.

Figure 3.11



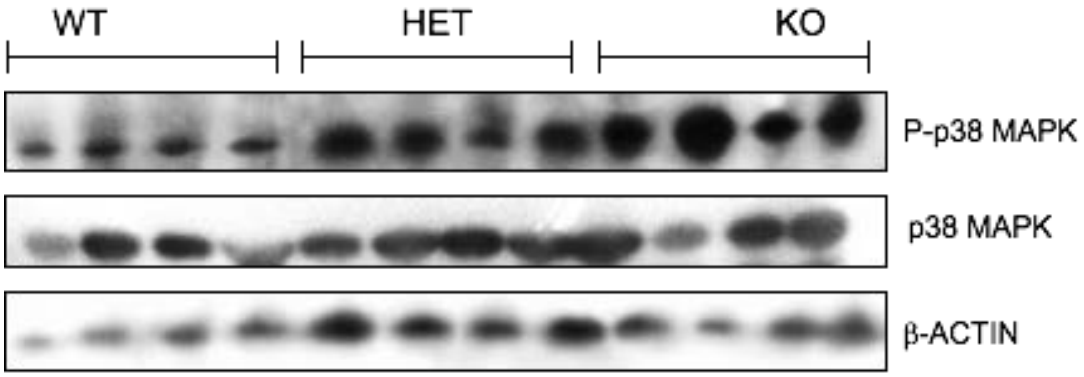
Densitometry of NST associated proteins in SUBQ. Densitometry of PPARy, PGC1a, and UCP1 proteins. WT: wildtype male littermates, HET: heterozygous male littermates, KO: knockout male littermates.

Figure 3.12



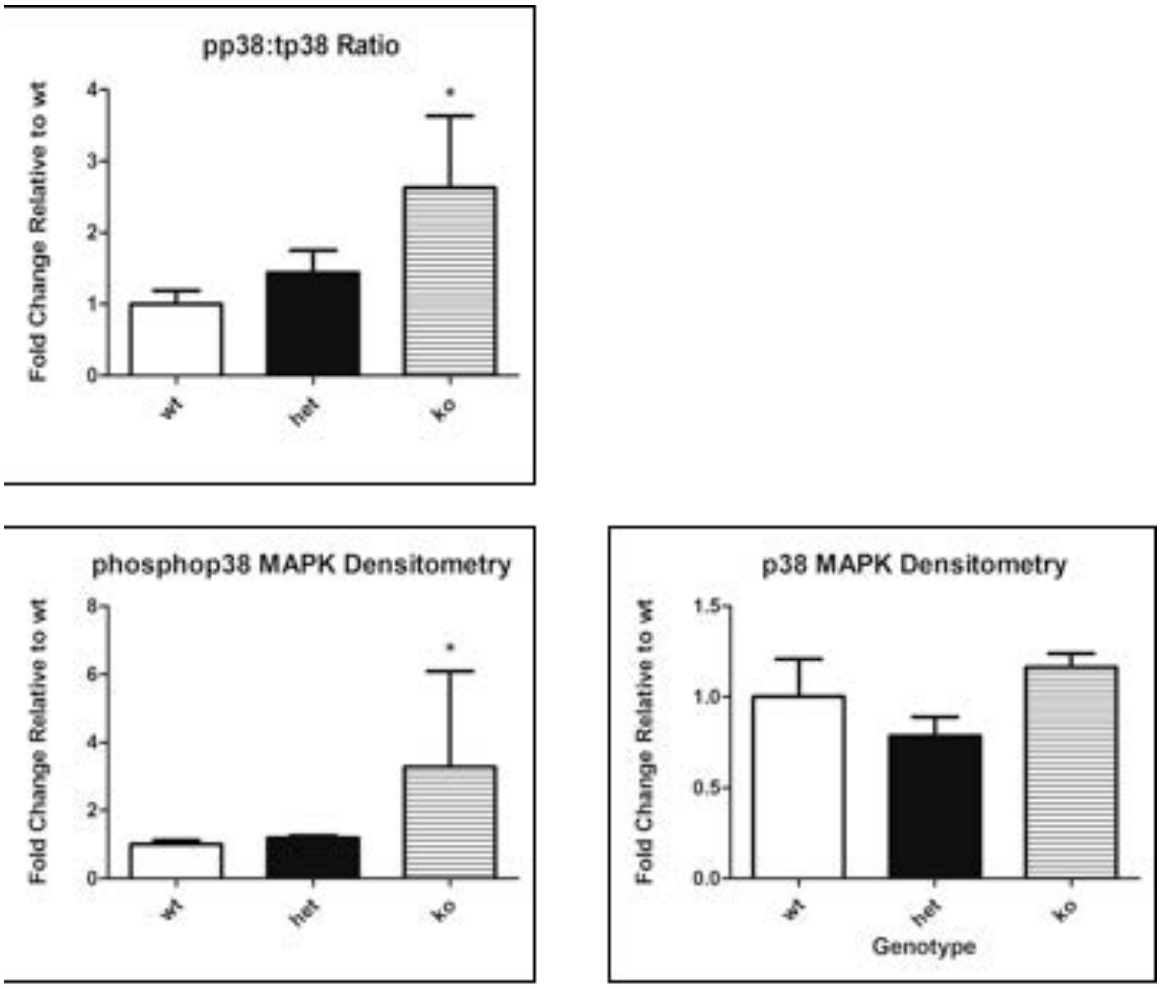
Subcutaneous adipose tissue of LIPA deficient mice increases UCP1 expression after cold exposure. Immunohistochemistry of UCP1 in inguinal SUBQ tissue sections of WT and KO mice after cold exposure. WT: wildtype male littermates, KO: LIPA deficient male littermates.

Figure 3.13



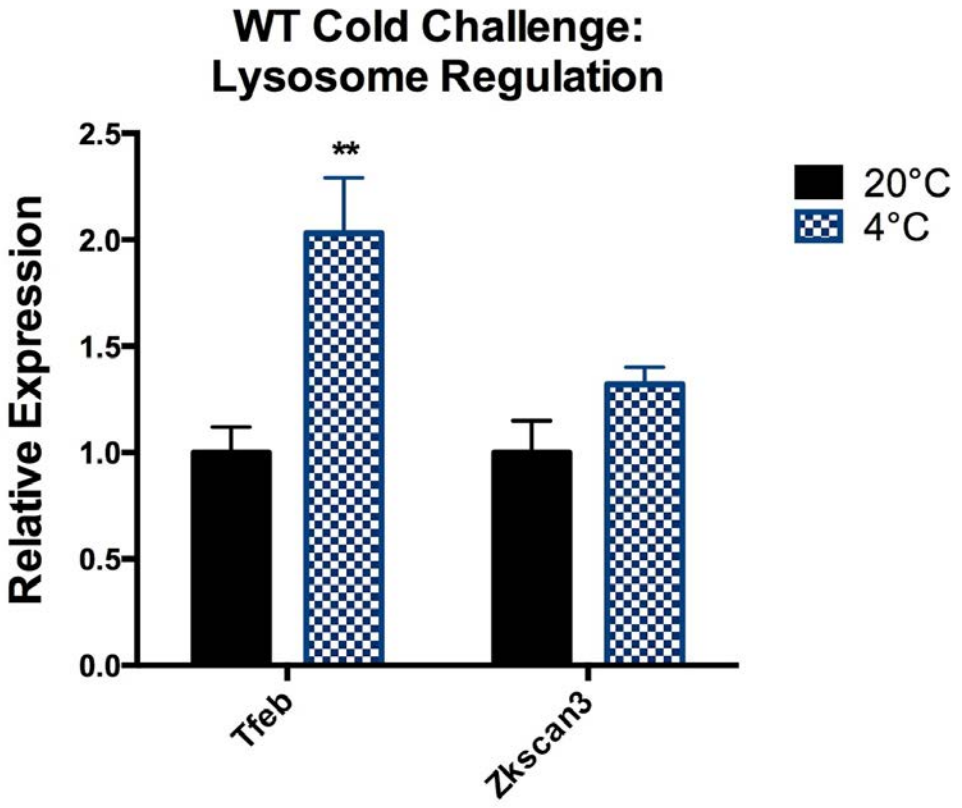
LIPA deficient mice show increased expression of p38MAPK in BAT. Immunoblots of phospho-p38 MAPK, p38MAPK and b-ACTIN. WT: wildtype male littermates; HET: heterozygous male littermates; KO: LIPA deficient male littermates.

Figure 3.14



Densitometry of phospho-p38 MAPK and p38 MAPK in SUBQ. Densitometry of phospho-p38 MAPK and p38 MAPK proteins. Pp38:tp38 ratio represents the densitometry ratio between phosphor-p38 MAPK and total p38 MAPK. WT: wildtype male littermates, HET: heterozygous male littermates, KO: knockout male littermates.

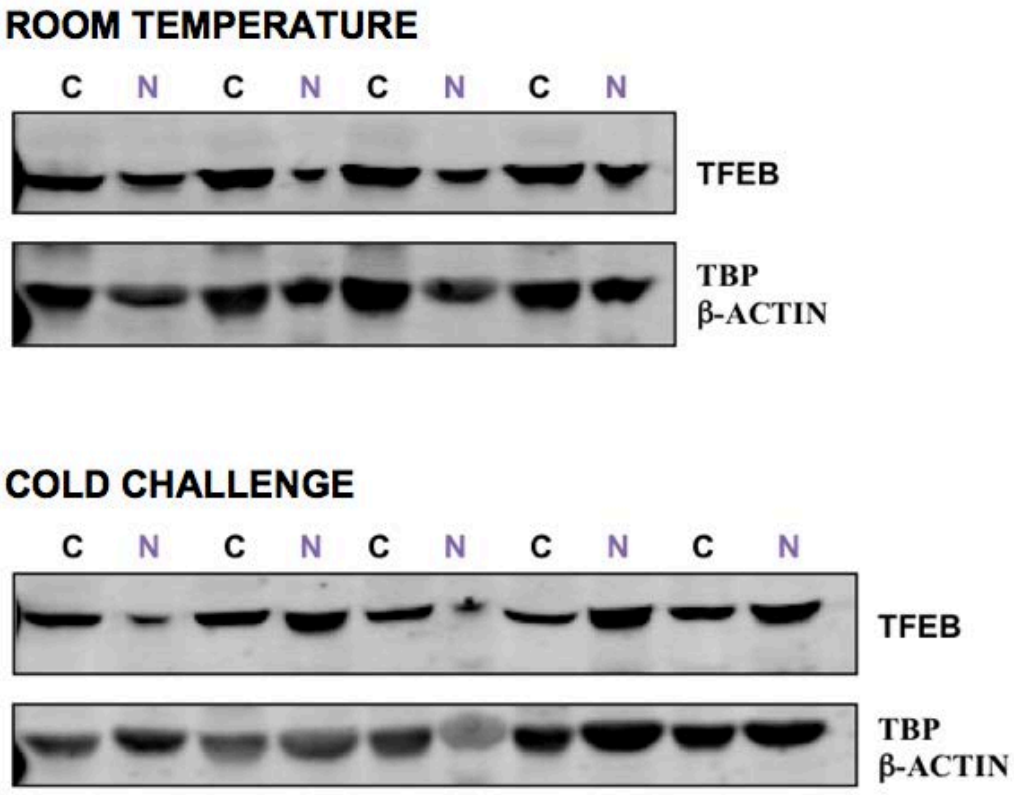
Figure 3.15



Cold challenge increases TFEB mRNA expression levels. Quantitative real time PCR of two major regulatory genes, *Tfeb* and *Zkscan3* after an eight hour cold challenge.

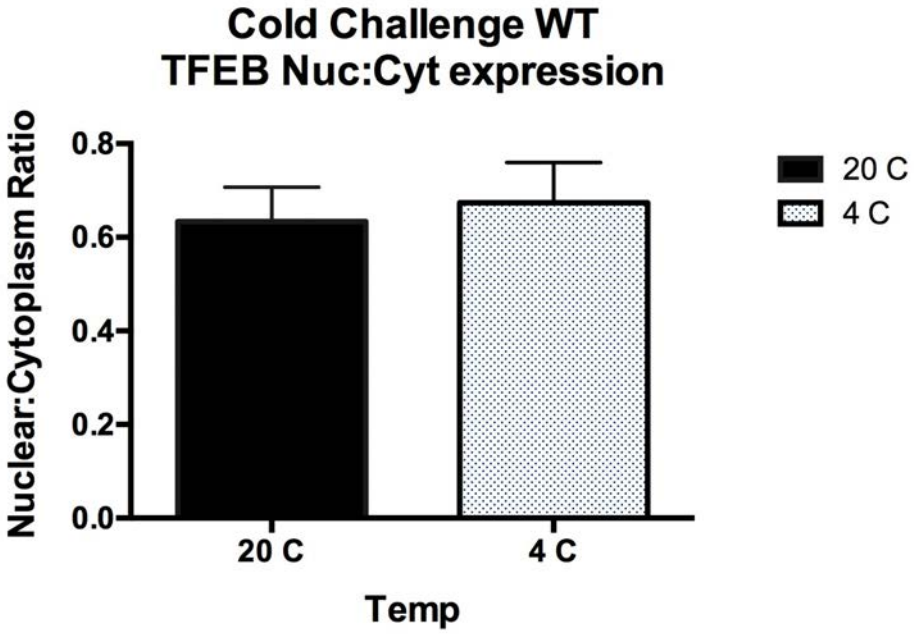
n = 5.

Figure 3.16



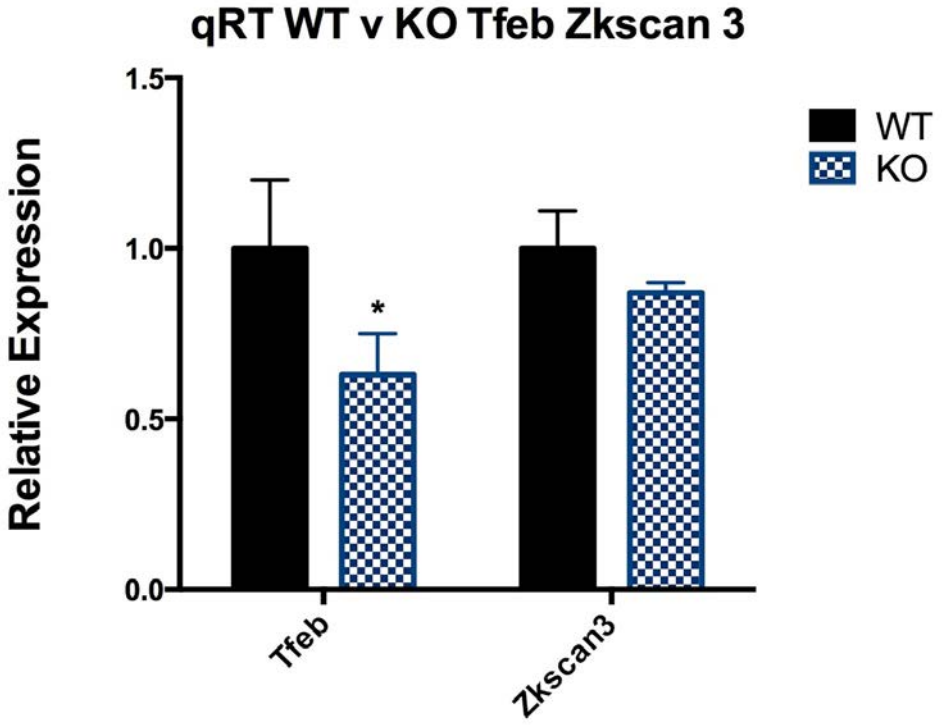
Cold challenge of wildtype mice does not alter TFEB translocation. Immunoblots of TFEB, TBP and β-actin of subcellular fractions from BAT depots of room temperature and cold challenged wildtype mice. C: cytoplasm; N: nucleus.

Figure 3.17



Cold challenge does not alter TFEB translocation in wildtype mice. Western blot of TFEB expression of nuclear and cytoplasmic fractions in room temperature or cold challenged wildtype mice.

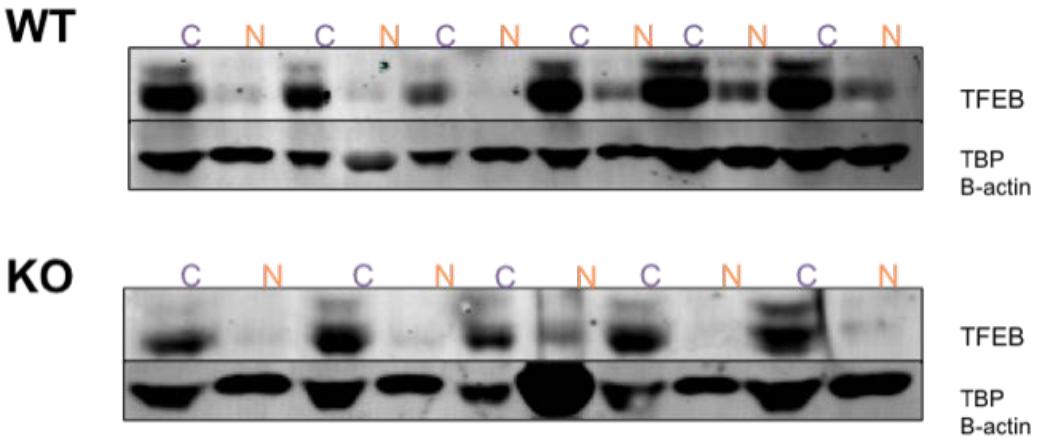
Figure 3.18



Cold challenged LIPA deficient mice have reduced TFEB expression compared to wildtype littermates. Quantitative real time PCR of two major regulatory genes, *Tfeb* and *Zkscan3* after an eight hour cold challenge. WT: wildtype male littermates; KO: LIPA deficient male littermates

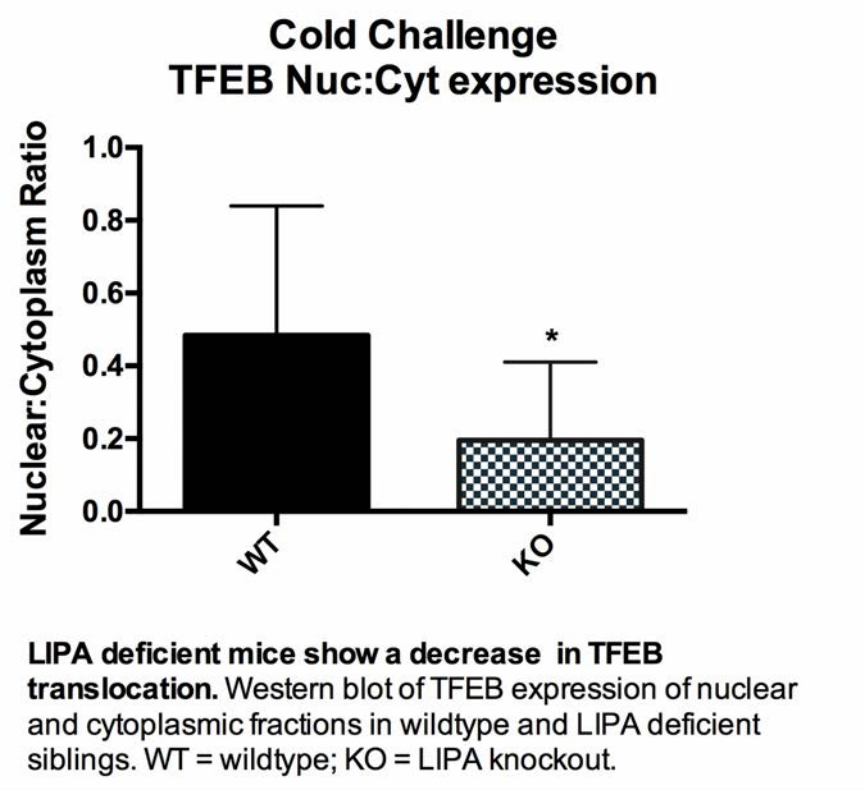
n = 5.

Figure 3.19



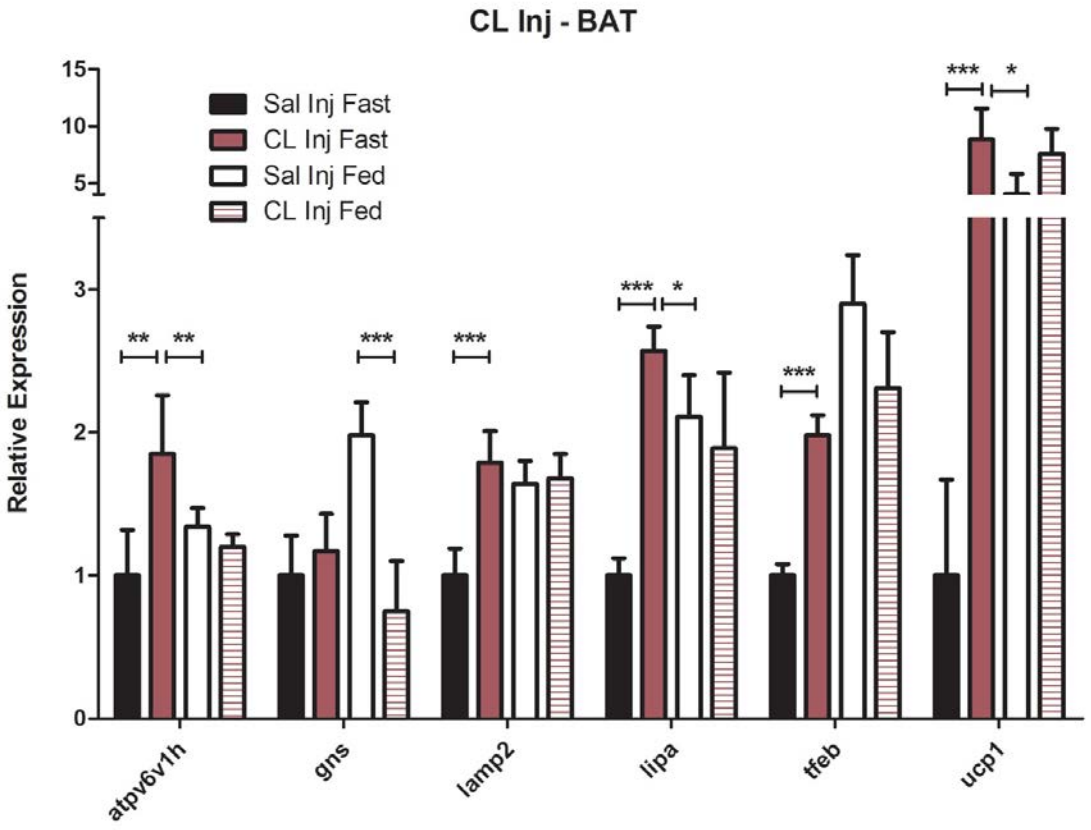
LIPA deficiency blunts BAT TFEB translocation in the cold. Immunoblot of TFEB, TBP, and b-actin in BAT of wildtype and LIPA knockout males after cold challenge. WT: wildtype male littermates; KO: LIPA deficient male littermates; C: cytoplasm; N: nucleus

Figure 3.20



LIPA deficient mice show a decrease in TFEB translocation. Western blot of TFEB expression of nuclear and cytoplasmic fractions in wildtype and LIPA deficient siblings. WT = wildtype; KO = LIPA knockout.

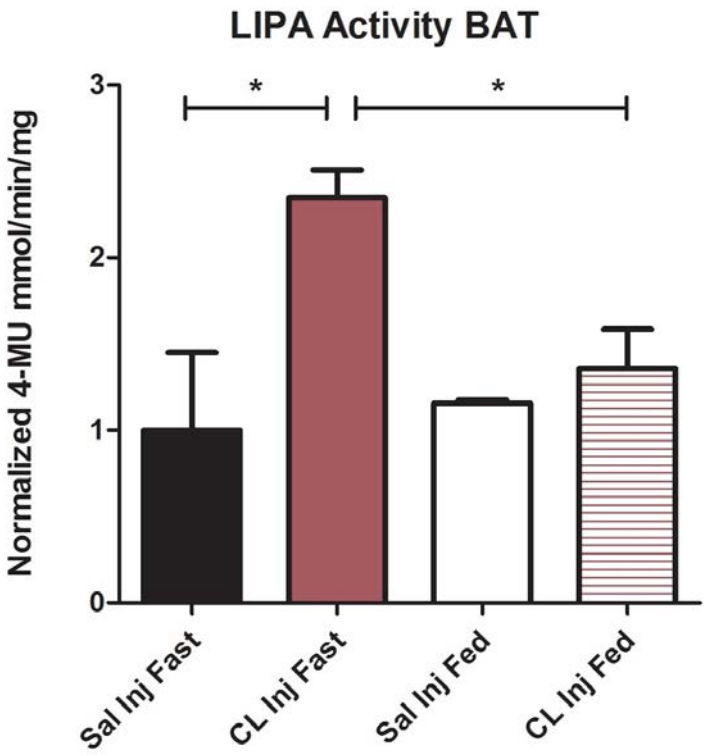
Figure 3.21



Lysosomal associated genes are upregulated when mice are both fasted and stimulated with a b3-AR agonist. Transcriptional activity in whole BAT tissue was measured by qRT-PCR and compared among saline fasted, saline fed, stimulated fasted, stimulated fed mice

n = 6.

Figure 3.22



LIPA activity increases only when mice are both fasted and stimulated with a b3-AR agonist. LIPA activity was measured by the 4-MUO method and compared amongh saline fasted, saline fed, stimulated fasted, stimulated fed mice.

n = 5.

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Settembre, Carmine, Rossella De Cegli, Gelsomina Mansueto, Pradip K. Saha, Francesco Vetrini, Orane Visvikis, Tuong Huynh, et al. 2013. "TFEB Controls Cellular Lipid Metabolism through a Starvation-Induced Autoregulatory Loop." *Nature Cell Biology* 15 (6): 647–58.

Settembre, Carmine, Alessandro Fraldi, Diego L. Medina, and Andrea Ballabio. 2013. "Signals from the Lysosome: A Control Centre for Cellular Clearance and Energy Metabolism." *Nature Reviews. Molecular Cell Biology* 14 (5): 283–96.

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CHAPTER FOUR

LYSOSOMES ARE THERMOGENIC ORGANELLES

Introduction

In the previous chapters, we found that thermogenic stimuli induce lysosome biogenesis in a tissue specific manner and that lysosome function is necessary for the full thermogenic response of mice to cold or febrile challenges. How lysosomes contribute to thermogenesis was not apparent from our initial experiments. We tested whether impairment of lysosome function altered canonical thermogenic signaling finding both inconsistent and modest results. In addition, the cold and LPS-induced lysosomal program was induced in nonUCP1 expressing tissue. This suggested another role for lysosomes in the thermogenic response.

As noted in the introduction, degradation of complex biomolecules will generate heat if there is catabolism of high energy bond but no capture of the energy. In our hypothesized model, lysosomes contribute directly to nonshivering thermogenesis as thermogenic organelles. Furthermore, beyond the heat generated from catabolic reactions, the acidification of the lysosome via V-ATPase requires the continual hydrolysis of ATP to ADP. By some calculations as much as 60% of the energy from this hydrolysis is released as heat. Moreover, any leak in this proton gradient would produce heat in manner similar to SERCA or UCP1.

The tools to directly test the ability of lysosomes to directly generate heat do not yet exist; here, we have attempted to develop techniques by which we could directly and quantitatively measure the heat generated by a cells. If successful,

these techniques would lay the foundations for methods to measure heat generated by organelles. We studied two methods: infrared thermography and isothermal microcalorimetry/isothermal titration calorimetry.

The least sensitive method for measure heat generation involves detection of infrared via a camera. Infrared tools measures and converts emissions of the infrared wavelength to quantifiable temperature readings. Infrared energy is emitted and absorbed by all objects as the molecules change their rotational-vibrational movements. The thermal radiation measured by infrared detection can thus be used as a surrogate for surface temperature. Infrared has been used for *in vivo* models of BAT thermogenic output (C. Jang et al. 2014; Crane et al. 2014; McCoard et al. 2014). While we attempted to use this same technology for *ex vivo* and *in vivo* experiments, we found that IR lacked accuracy, precision, and sensitivity.

Another method, isothermal microcalorimetry (ITC) proved more robust and less susceptible to environmental effects and could measure quantitatively the heat generated by suspensions of cells. Isothermal microcalorimetry (IMC) is a powerful method that allows for real-time monitoring and analysis of chemical, physical, and biological reactions (Koenigbauer et al. 1992; Willson et al. 1995; Charlebois, Daniels, and Lewis 2003; Lewis, Gladius, and (Dan) Daniels 2003). IMCs have been used to determine prokaryotic and eukaryotic cell growth and count in medium (Monti 1990a; Braissant et al. 2010/1; Rodríguez et al. 2011); we chose to use this same type of technology to measure heat production from our cells. As a form of IMC, we used an isothermal titration calorimetry (ITC) system as our primary instrument. This type of microcalorimetry is specifically designed to determine the thermodynamics of reactions within a solution. Most

often used to study binding affinity, enthalpy changes, and binding stoichiometry in biochemical reactions, it tends to be more sensitive than other IMC systems and is well suited for our studies due to its application for thermodynamics (Pierce, Raman, and Nall 1999).

The central principle governing ITC technology is dependent on detecting temperature differences between two "cells" (not to be confused with the biological definition of the word). Within the instrument, there is a reference and a sample cell composed of thermally conducting and chemically inert material. The reference cell is filled with water while the sample cell contains a solution generally containing a macromolecule for binding studies. A power is continuously applied to the reference cell to heat the cell at a predetermined, specified temperature. Simultaneously, a feedback circuit also activates a heater in the sample cell. When a titrant is added into the sample cell at specific volumes, the reaction within the cell will either be exothermic or endothermic. Heat will hence either be released or taken up, causing the feedback circuit applied to the sample cell to be either decreased or increased to maintain an isothermality between the reference and sample cell (Fig 4.1). The amount of input power per unit of time to maintain equal temperatures is measured and plotted. The resulting figure should resemble that of Figure 4.2 for our *ex vivo* and *in vitro* experiments.

Using these calorimetric techniques, this chapter explores not only novel methods of studying thermogenesis in cells but also the potential thermogenic nature of lysosomes using indirect approaches to alter lysosomal function.

Hypothesis and proposed model:

Lysosomes are thermogenic organelles that contribute to overall homeothermy.

Methods and Materials

Isothermal titration calorimetry.

ITC measurements were performed on two instruments, Malvern's VP-ITC (courtesy of the Schramm lab at Albert Einstein College of Medicine) as well as Malvern's Auto-ITC200. Reference cells were filled with degassed water, while the sample cells were filled with cell medium. The ITC syringes were loaded with cell medium and isolated cells in single cell suspension, degassed for 20 minutes prior to loading. Parameters of the ITCs were as follows: Two injections, jacket temperature of 36 C, stirring speed of 200 rpm. Reference power was set at 15 for the VP-ITC instrument but at 5 for the Auto-ITC200. Due to the difference in volume capacities, injection volumes for the VP-ITC machine were: 8 ul, 200 ul (unless otherwise specified) while the injections volume for the Auto-ITC200 were: 3ul, 35 ul. Raw data in the form of uCal/sec and time were collected and plotted in Graphpad's Prism software. Change in power were determined by taking the difference of averages of ten time points prior and ten points post the second injection. This change compared between the control and experimental group and significance was determined using the standard student t-test.

Brown adipose tissue isolation.

Brown adipocytes were isolated from extracted whole interscapular brown adipose tissue. Tissue were minced such that individual pieces were ¡1 mm or less in diameter in 350 ul of cell medium, resulting in a tissue slurry. Following mincing, the tissue slurry was placed in cell medium with liberase (Roche) and incubated at 37C with shaking for 30-50 minutes, depending on the efficiency of the liberase stock. The resulting solution was passed through a 100 micron cell strainer (Gibson) and centrifuged at 500g for 10 minutes. The supernatant was removed and the cells were reconstituted in 5 mLs of cell medium before being

centrifuged again at 500 g for 10 minutes. Cells were then reconstituted in cell medium and counted for cell concentration.

Splenocyte isolation.

Splenocytes were isolated from whole spleens extracted from sacrificed mice. Following extraction, splenocytes were placed in a plate containing a 70 micron cell strainer immersed in cell medium. A 10 mL syringe plunger was used to press the whole spleen within the cell strainer against the plate wall. After filtering the ground tissue through the cell strainer, cells were centrifuged at 500g for 5 minutes. Following centrifugation, cells incubated in a lysis buffer for five minutes at room temperature (Invitrogen) to lyse the erythrocytes. The suspension was then pelleted again via centrifugation at 500g for an additional 5 minutes before resuspending again in two mLs of cell medium. Cells were counted for concentration prior to ITC measurements.

Chloroquine injections.

Chloroquine (Sigma-Aldrich), a lysosome inhibitor, was injected either interperitoneally or directly into the BAT tissue at 3.5 mg/kg.

CL 316,243 injections.

CL-316,243 (Sigma-Aldrich), a beta3-adrenergic receptor agonist, was injected interperitoneally at 1 mg/kg. Mice were sacrificed 2 hours post injection.

Chloroquine ex vivo treatment.

Chloroquine (Chloroquine diphosphate salt, Sigma-Aldrich) was dissolved in sterile water at a concentration of 100 nM. 100 uL of 100 nM chloroquine was injected subcutaneously in the interscapular region of mice. Littermate controls

were injected with 100 uL of sterile water. Mice were rested for two hours before subsequent procedures were performed.

Statistics.

Significance calculations of comparisons between two groups were determined by student's t-test. Significance calculations of multiple group comparisons were determined first by ANOVA and then Bonferroni correction. Key for all significance indications: * = p-value ≤ 0.05; ** = p-value ≤ 0.01; *** = p-value ≤ 0.001.

Figures represent one experiment out of four. Data are not combined to produce the figures or statistics. Error bars represent the standard deviation.

Results

Isothermal calorimetry can be used to measure heat production by primary cells.

To assess the contribution of lysosome function to thermogenesis and confirm that alterations, we sought methods to quantitatively measure heat generated by cells. In our first attempts to quantitatively measure heat generation, we used an infrared camera in a manner similar to what others had done (Lee et al. 2014; Christina Jang et al. 2014). However, we found it inadequate in several ways: (1) Infrared cameras measure surface temperature and therefore are affected by ambient temperature and airflow; (2) the surface temperature of a well containing cells is also dependent upon the number of cells and the geometry of where the cells sit in the well; and (3) edge effect dissipation greatly confounded our readings.

An alternative approach is to measure the heat generated by a closed chamber using isothermal titration calorimetry (ITC), a form of isothermal microcalorimetry. ITCs are used primarily for binding assays, measuring enthalpy of interactions in solution. ITCs are capable of measuring in the nanocalorie range, sensitive enough to make it a potential technique to determine lysosomal heat contribution. The similar calorimetric technique, isothermal microcalorimetry (IMC), can be used to determine cellular growth and decay overtime (Monti 1990b; Rodríguez et al. 2011; Braissant et al. 2010/1). ITCs have not been shown to work with cellular biology applications.

To assess the applicability of ITCs for our experiments, we first used the technique to measure cellular heat production and gradually increased cellular

concentration within the sample ampoule. We reasoned that if ITC would be useful than there should be a linear relationship between the number of cells and heat generated. To test this we measured heat produced by $1x10^3$, $2x10^4$, $2x10^5$, and $1x10^6$ splenocytes (Fig 4.3 and 4.4). Splenocytes were chosen for the pilot procedure due to their ease of isolation. We found that there was a relative linear relationship between heat generation (measured power - microcalories/sec) and the number of cells (Fig 4.4).

If ITC could measure meaningful changes in heat, we further reasoned that adrenergic stimulated BAT should generate measurably more heat than nonstimulated cells. We measured heat generated by 2.5 x 10^5 cells kept at basal (medium + PBS) or thermogenic (medium + CL) condition for two hours prior to placement in the ITC. At baseline and 37˚C, BAT cells produced 1.7 picocalories/sec/cell and, upon stimulation with CL, produced 6.0 picocalories/sec/cell) (Fig 4.5 and 4.6). These data provide, to our knowledge, the first direct quantitative measurement of BAT heat production and the effects of activation with adrenergic stimulation. Together, these data provide evidence that we could quantitatively measuring heat production using ITC.

Heat production by lysosome impaired BAT and nonUCP1 murine tissues

Because our tissue samples were derived from mice already acclimated to room temperature, about 5-10˚C below thermoneutrality, sympathetic activation of BAT already exists in our UCP1 tissues. We expected that this activation would result in higher baseline heat production in BAT compared to non-UCP1 tissues. We compared the heat produced by $1x10^6$ cells isolated from BAT and

splenocytes. We did indeed find that BAT cells produced approximately 3.1x more heat than splenocytes (Fig 4.7 and 4.8).

To test whether impairment of lysosome function would reduce heat generation we treated cells with chloroquine (CQ). In wildtype, unstimulated BAT chloroquine reduced by 40% heat production (Fig 4.9 and 4.10). Similarly, BAT from *Lipa-/-* mice produced 60% less heat compared to BAT from *Lipa+/+* mice (Fig 4.11 and 4.12).

While we found differences in the baseline calorimetric readings between wildtype or control and LIPA deficient or CQ treated tissue, adrenergic stimulation may override any baseline differences. However, we found that adrenergic stimulation by CL-316,243 was unable to restore full thermogenic capacity of either LIPA KO BAT (Fig 4.13) or CQ injected BAT (Fig 4.14). These findings are consistent with the in vivo finding that *Lipa-/-* suffer from more severe hypothermia than CQ treated mice and .support the importance of functional lysosomes in thermogenesis.

Impaired lysosomal function reduce thermogenesis in a nonUCP1 tissue

The induction of lysosome biogenesis in nonUCP1 tissues in response to cold and febrile stimuli suggested that lysosomes may provide an adaptive role in maintenance of body temperature. To test directly whether impaired lysosome function directly reduces heat production, we measured heat generated by splenocytes from wildtype mice treated with PBS or with CQ. Treatment with CQ reduced heat production by 21% (Fig 4.15). Similarly, splenocytes from *Lipa-/-* mice produced 34% less heat than splenocytes from *Lipa+/+* mice (Fig 4.16

and 4.17). These findings argue that lysosomes contribute significantly to the generation of heat by both UCP1 expressing and non-expressing cells.

Summary and Proposed Model

Lysosomes contribute to body temperature maintenance through heat production (Fig 4.18).

Conclusions

The conclusions of this chapter are twofold. First, we established that isothermal microcalorimetry, in particular, isothermal titration calorimetry, can be used to measure heat production from cells, including brown adipocytes and splenocytes. Second, we provide evidence that functioning lysosomes contribute substantially to basal and induced thermogenesis in UCP1 expressing and non-expressing tissues.

While we had some preliminary data from infrared (IR) technology, we realized that IR was limited by a number of factors. First, edge effect altered accuracy. Second, IR readings measured surface readings, affecting both accuracy and precision. Finally, sensitivity of IR measurements was limited to only 0.1˚F/C. Therefore, a better alternative to measure heat production was needed.

To demonstrate the utility of ITCs for thermogenesis-related studies, we performed a number of negative and positive control tests. Negative controls using only cell medium initially showed that our readings are not artificial. Non-thermogenic cells were also able to produce modest amounts of heat, which increase proportionally with cell number. Additionally, as one would predict, cells from BAT produced more heat than cells not dedicated to thermogenesis. We further examined differences in heat production from brown adipocytes with and without activation by the β3-adrenergic receptor agonist, CL-316,243. This positive control further convinced us of the utility of ITCs. Taken together, these data help establish ITC as a method to quantitatively measure cellular heat production and that it is particularly suitable for thermogenic studies *ex vivo*.

Convinced of the utility of ITC, we used our newly established methods to test our hypothesis that lysosomes are thermogenic organelles. Similar to our earlier experiments, we used both pharmacologic and genetic approaches to disrupt lysosomal function. Both chloroquine treatment and LIPA deficiency resulted in decreased heat generation from brown adipocytes. A similar deficit in heat production was also found when lysosome function was impaired in adrenergically stimulated BAT. Perhaps more intriguingly however, the same approaches resulted in a small but statistically significant decrease in basal heat production from a "non-thermogenic" cell type, the splenocyte. These data now provide functional evidence that the induction of lysosome in response to cold and febrile challenges contributes directly to the generation of heat by both UCP1 and non-UCP1 tissues.

Limitations and Concerns

Ex vivo data.

The *ex vivo* model we used to explore our thermogenesis has both advantages and disadvantages. As the study of temperature regulation is a whole body phenomenon, integration of multiple systems is vital to maintain body temperature. Studying cells in isolation may therefore over- or under- estimate the contribution of any manipulation to the overall maintenance of body temperature. However, by isolating primary cells from our mouse models, we were able to determine whether alterations in lysosome function had a direct effect on thermogenesis independent of any heat exchange processes.

Tissue heterogeneity.

Again, the heterogeneous nature of our tissues brings questions to our interpretation of the data. We do not know, for example, which cells from BAT and spleen are responsible for the thermogenic measurements we made with ITC. However, the extra steps necessary to fully isolate the adipocytes from this fraction would be an additional confounding component that could influence our data. Our ultimate goal in this chapter is to demonstrate that lysosomes are capable of generating heat, regardless of its tissue or cell of origin. As such, tissue heterogeneity may be more a tertiary concern compared other chapters.

Controls.

While we used both dead cells and cell medium as our negative controls, the ideal negative control for our CL-316,243 activation studies would come from UCP1 ablated tissue. Heat production from UCP1 ablated tissue would also represent thermogenesis from a UCP1 independent manner, possibly contributed by lysosome action. Unfortunately, we were not able to obtain the UCP1 knockout

mice in time for this study and is included in the *"Future Directions"* section of the final chapter to this dissertation.

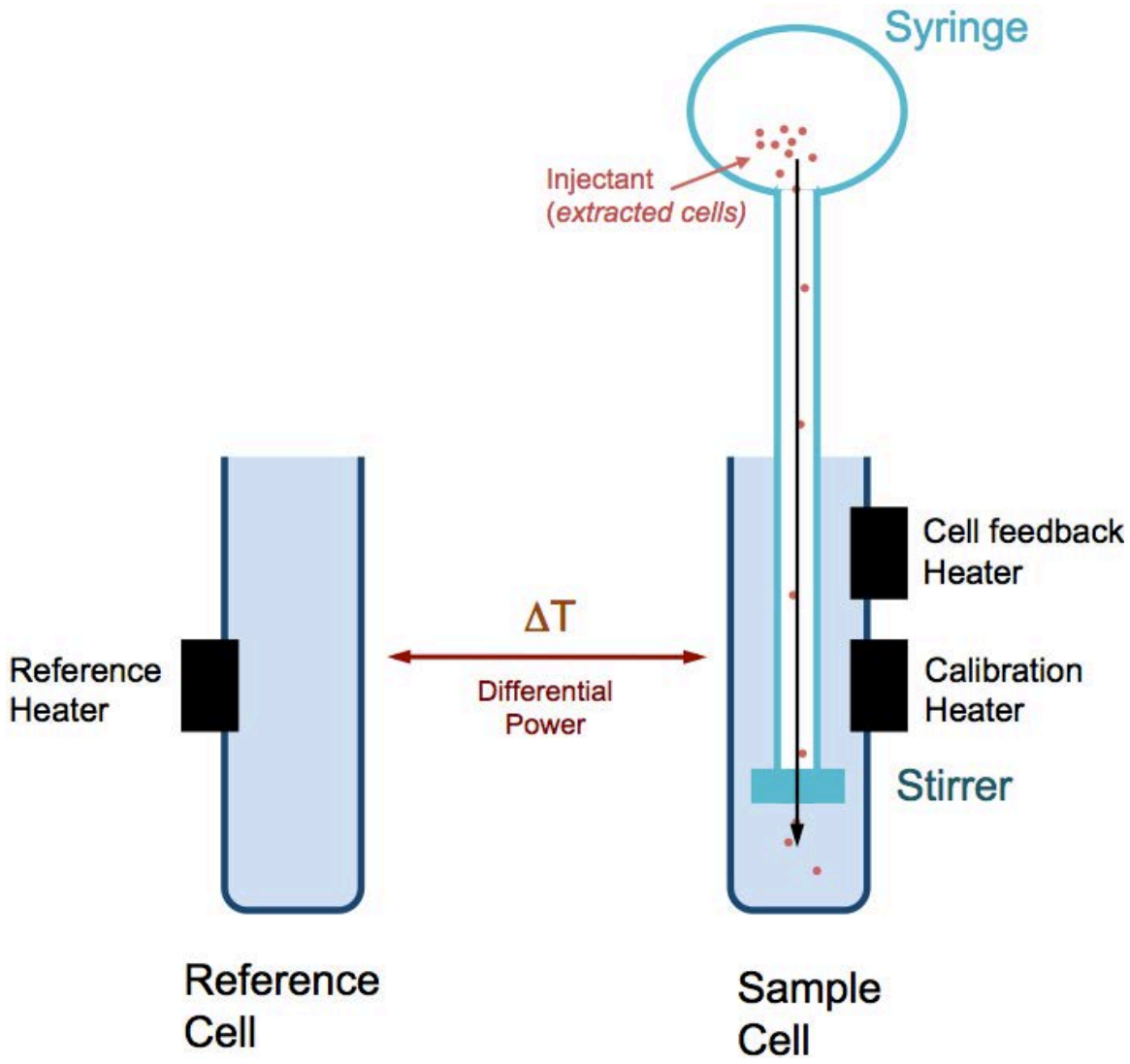
Indirect data. One of the primary goals of this chapter is to show that lysosomes could be thermogenic organelles. Unfortunately, all of our data are indirect, using pharmacologic and genetic agents. Although some of this was circumvented by using UCP1 negative tissues, other components may still be at play. Both the pharmacologic and genetic approaches could change cell populations as well as contribute to overall cellular dysfunction. Additionally, in the BAT tissue, coupling between the lysosome and the mitchondrion would disguise our data. To address this concern, we would ideally isolate lysosomes directly to determine the direct heat contribution of the organelle. This is also discussed further in the "*Future Directions"* section of the final chapter.

Technical.

ITCs require nearly an hour of time to equilibrate and wash prior to measurements. This excess hour can severely affect our observed phenotype as the container holding our medium with cells is not optimized for cell culture. As an *ex vivo* study, the same limitations discussed in the *ex vivo* section would only be compounded with this delay. Further, because measurements of multiple samples can not be measured simultaneously, environmental factors such as temperature fluxes, circadian regulation, and human error would be compounded as samples are measured individually.

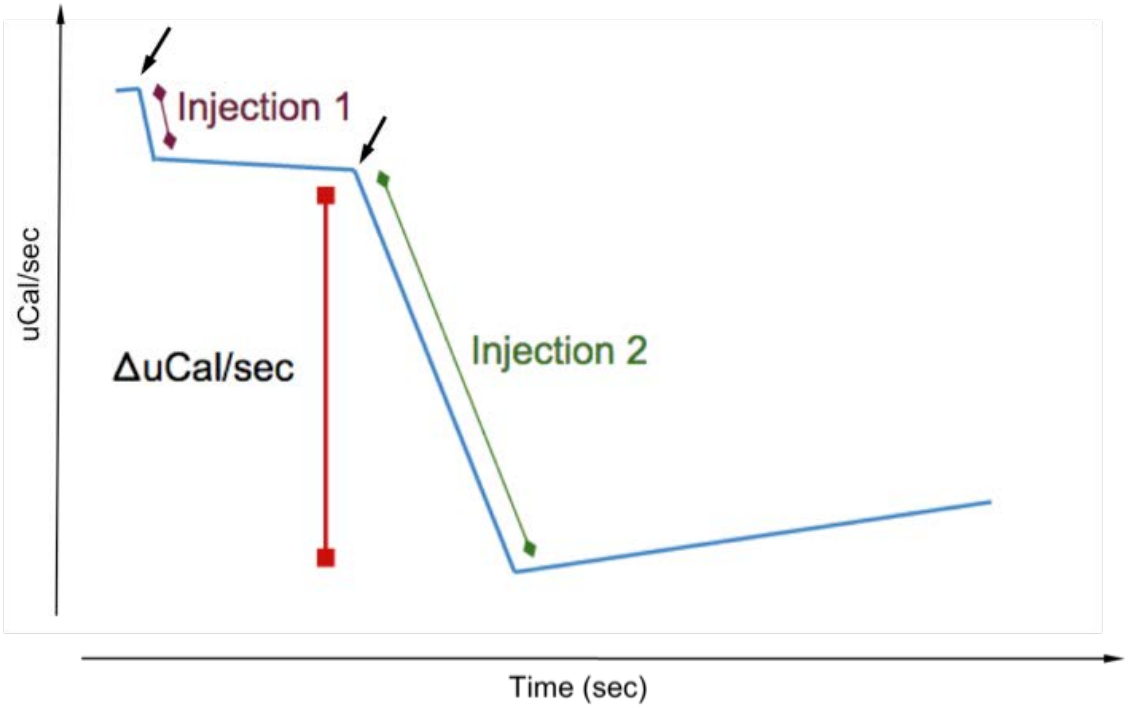
Figures

Figure 4.1



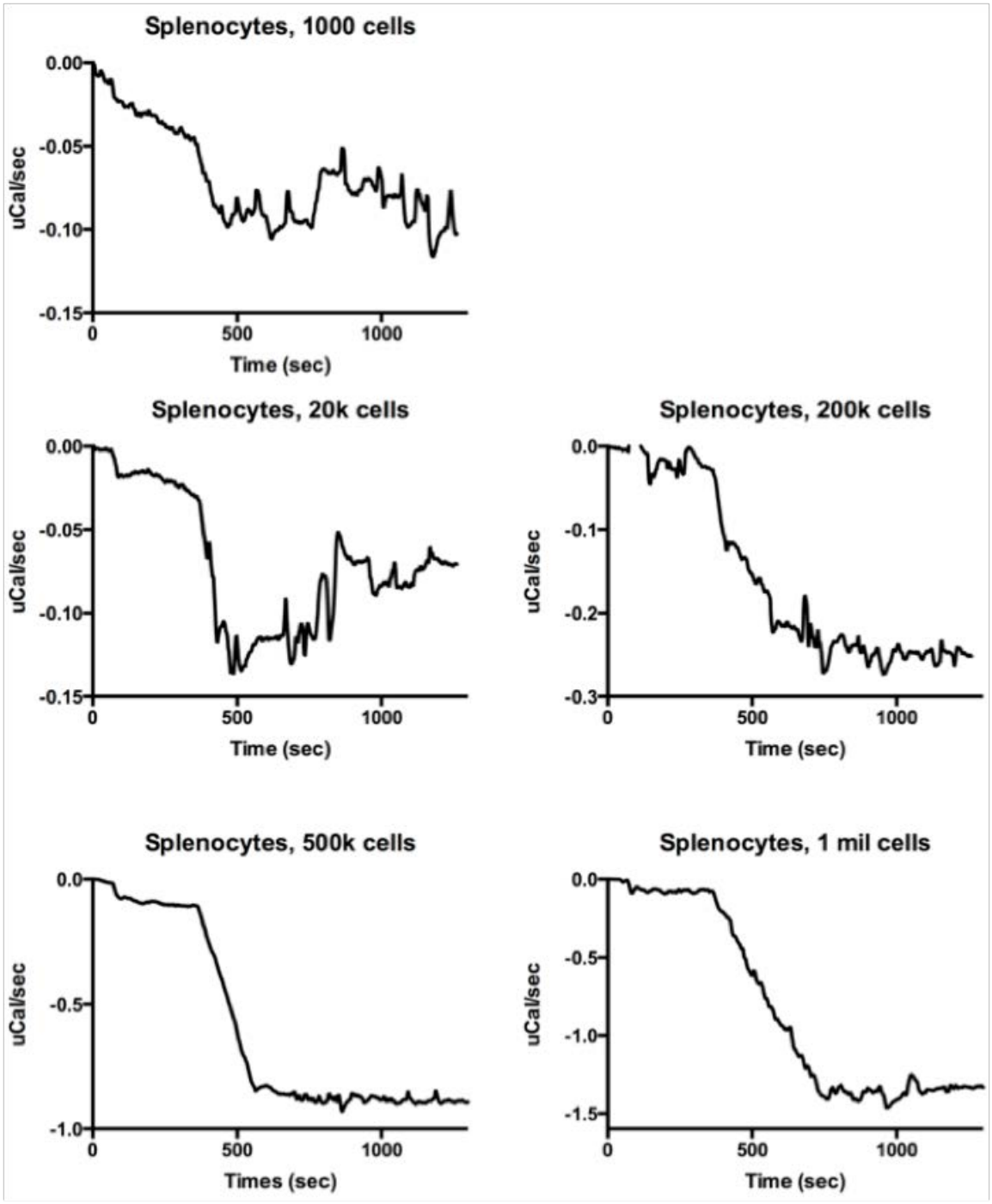
Isothermal calorimetry instrumentation setup.

Figure 4.2



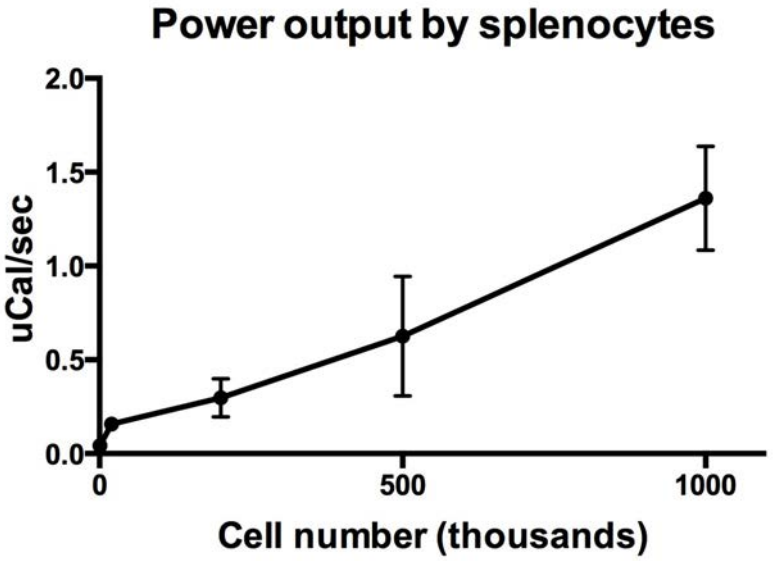
Schematic of anticipated ITC results. Arrows represent the start of injections. The first injection is a small volume and will result in a minor decrease in power input. The hold time after the first volume establishes the initial baseline reading. A longer and greater volume is injected, resulting in a large decrease in power input. The amount of power released by the injectant is determined by the difference in the average power measurement of the first hold period and of the second hold period.

Figure 4.3



Increasing splenocyte number decreases power input to ITC sample cell. ITC uCal/sec measurements of isolated splenocytes at increasing concentrations. Representative graphs, n=1 per graph.

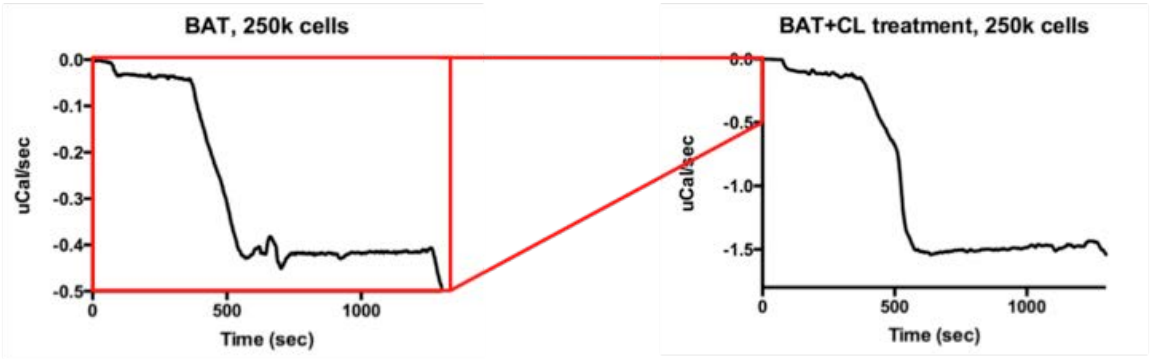
Figure 4.4



Increasing splenocyte number results in a subsequent increase by power. Quantification of power (uCal/sec) measurements determined by ITC plotted against splenocyte cell number.

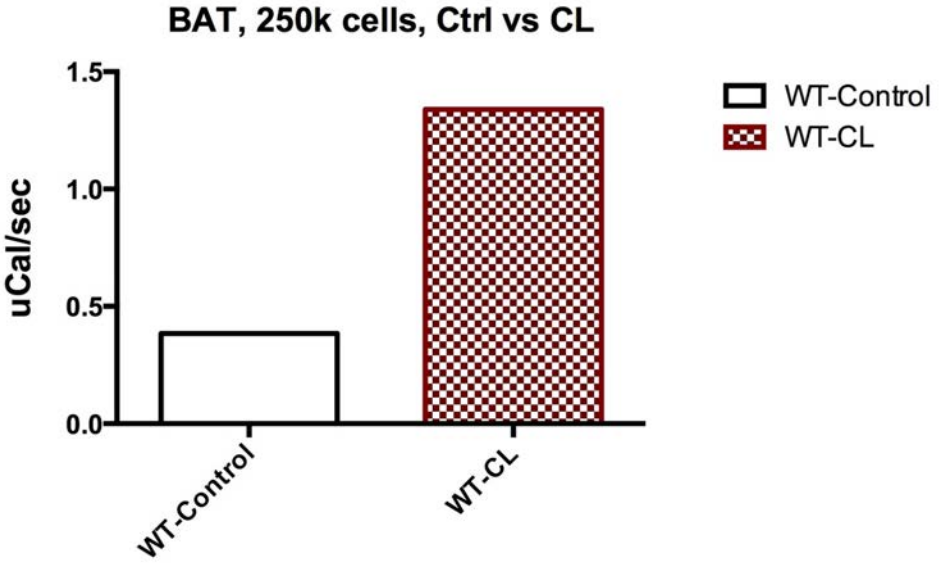
n = 3

Figure 4.5



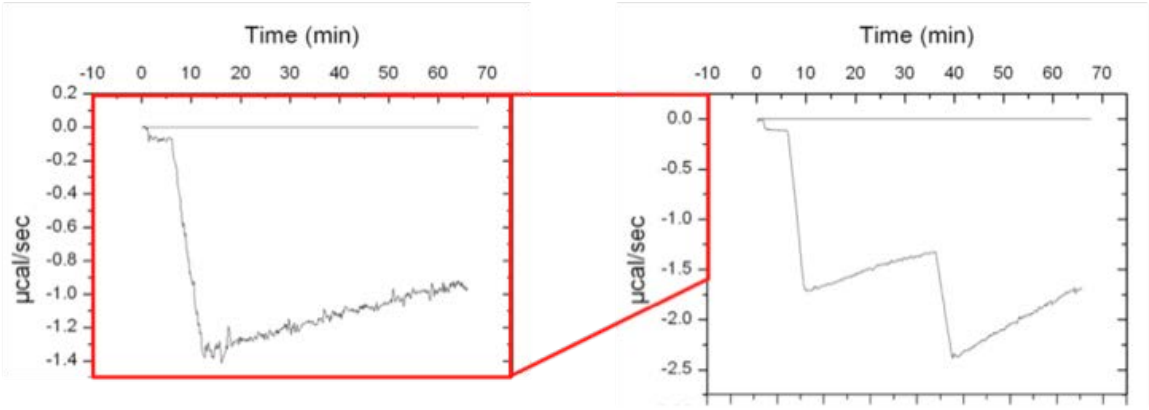
CL-316,243 treatment increases heat production by isolated BAT. Isolated cells from BAT depots were treated with either PBS (left) or 100 nM CL-316,243 (right) for two hours. CL: CL-316,243 treated. Representative graphs, n = 1 per graph.

Figure 4.6



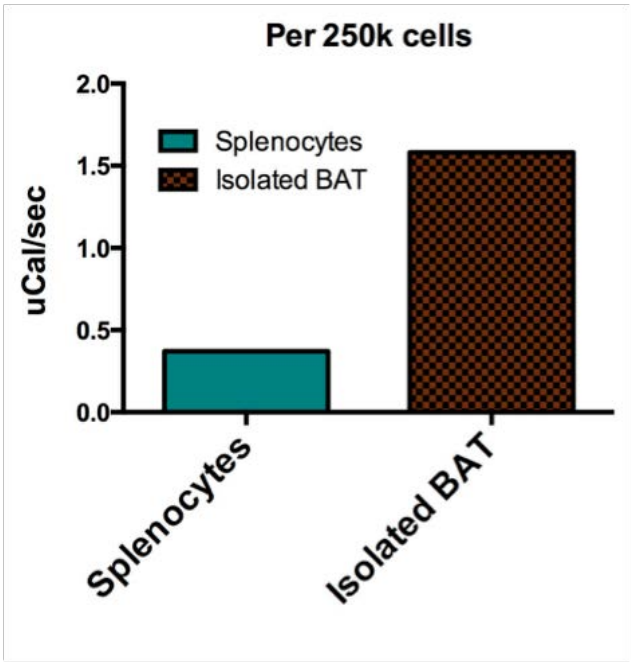
CL-316,243 treatment increases heat production by isolated BAT. Column representation of uCal/sec ITC readings of control treated vs CL-stimulated isolated BAT cells. Ctrl: Control treated; CL: CL-316,243 treated.

Figure 4.7



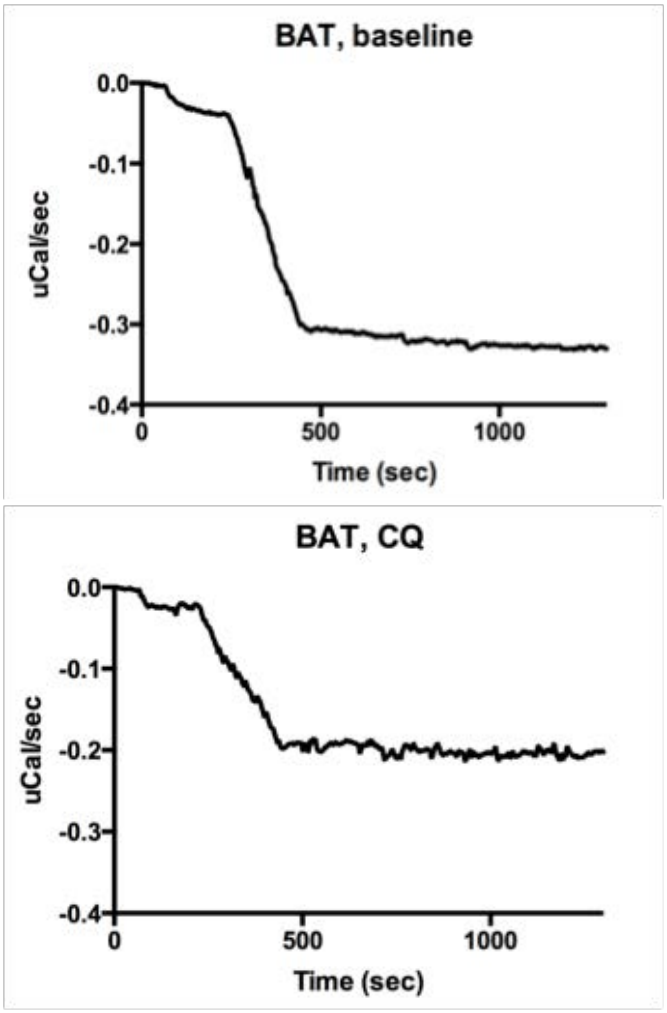
Isolated BAT cells produce more heat than isolated splenocytes. Isolated cells from BAT depots compared with isolated splenocytes.

Figure 4.8



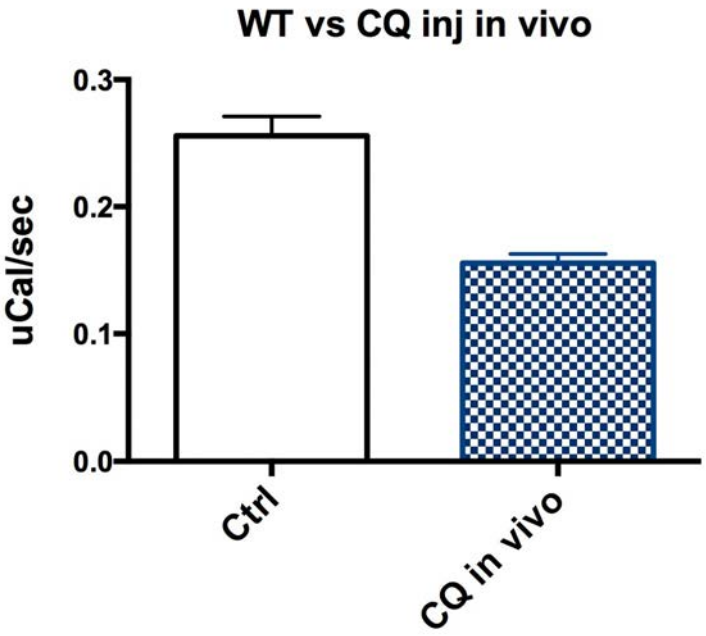
Isolated BAT cells produce more heat than isolated splenocytes. Isolated cells from BAT depots compared with isolated splenocytes; power adjusted per 250k cells.

Figure 4.9



Chloroquine treatment reduces isolate BAT heat production. Isolated cells from BAT depots comparing control injected versus CQ injected mice. Baseline: control; CQ: chloroquine injected. Representative graphs; n=1 per graph.

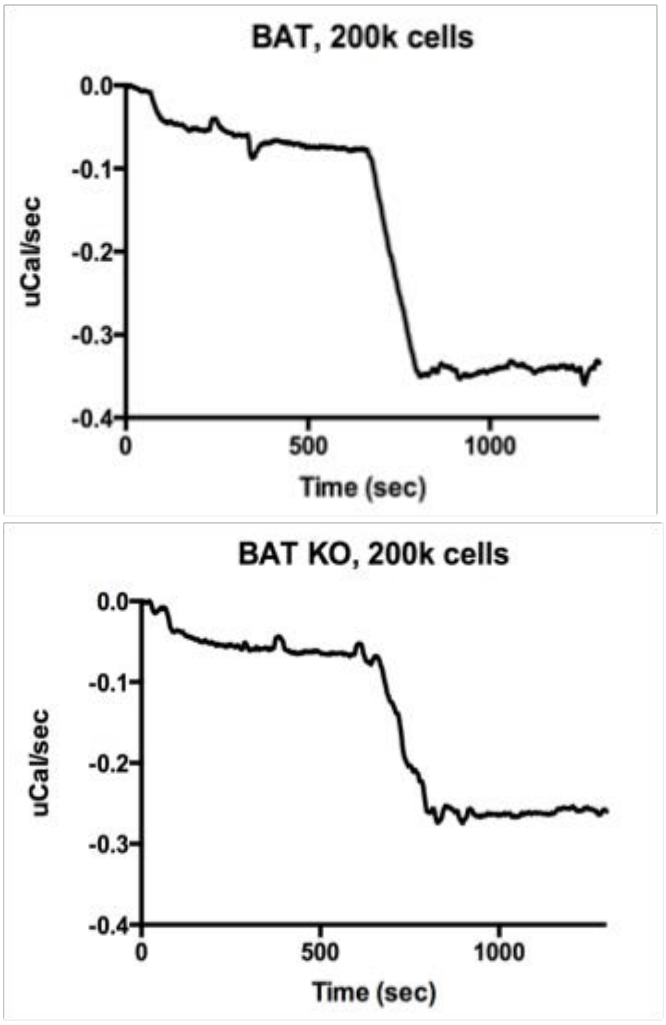
Figure 4.10



Isolated BAT cells treated with CQ produce less heat.
Graphical representation of control or CQ treated BAT. Ctrl: control; CQ: chloroquine.

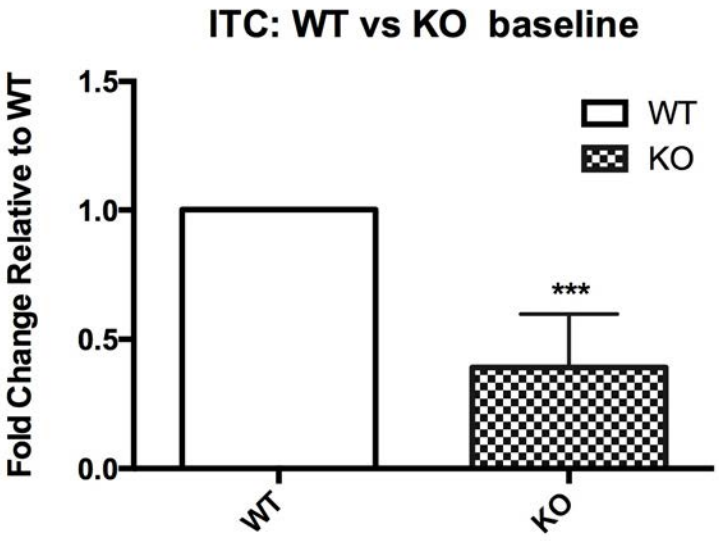
n = 5; p-value <0.01.

Figure 4.11



LIPA deficient BAT produce less heat than wildtype counterparts. Isolated cells from BAT depots comparing wildtype and LIPA deficient littermates. Left: wildtype isolated BAT cells; Right: LIPA deficient isolated BAT cells. Representative graphs; n=1 per graph.

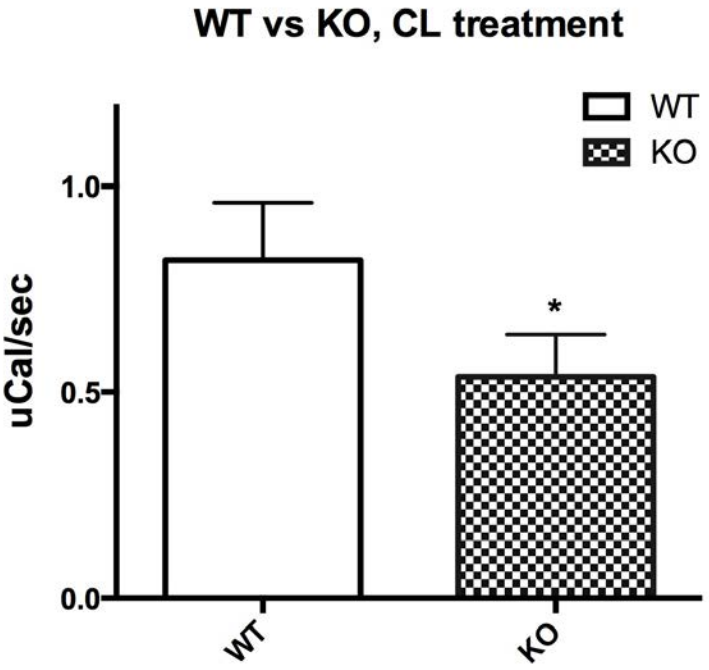
Figure 4.12



LIPA deficient BAT produce less heat than wildtype counterparts. Heat fold change between wildtype and LIPA deficient isolated BAT cells. WT: wildtype male littermates; KO: LIPA deficient male littermates.

n = 5.

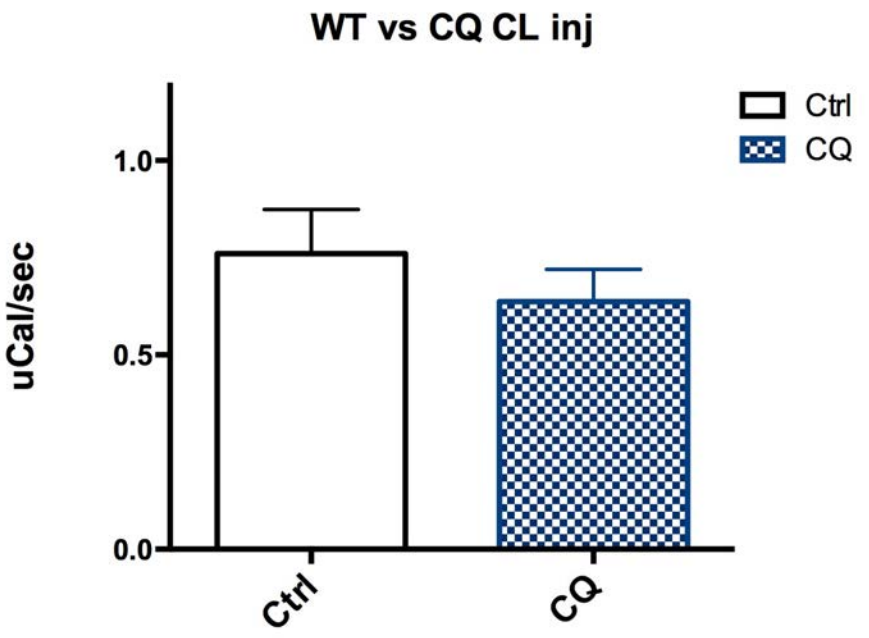
Figure 4.13



LIPA deficient mice are less responsive to adrenergic thermogenic stimulation. ITC measurements of isolated BAT cells from CL-316,243 IP injected (1 mg/kg) mice. WT: wildtype male littermates; KO: knockout male littermates.

n = 4.

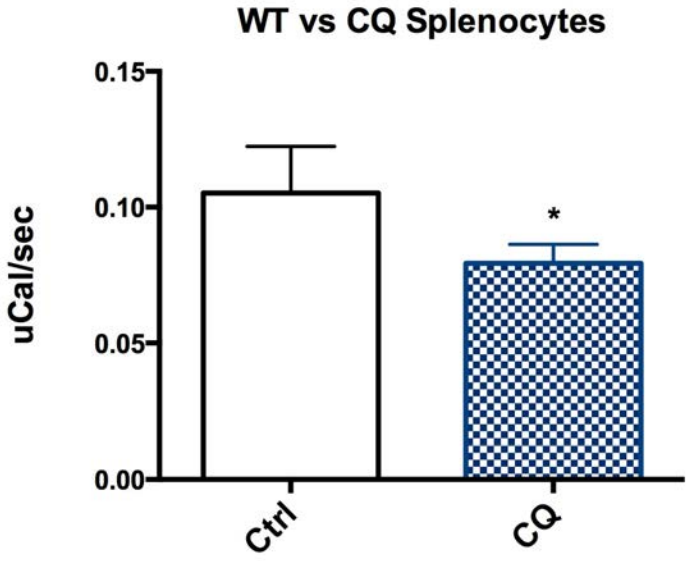
Figure 4.14



Heat production of adrenergically stimulated isolated BAT cells of control and CQ injected animals. ITC measurements of isolated BAT cells from control and CQ (3.5 mg/kg) treated mice. Ctrl: control (saline) injected male littermates; CQ: chloroquine injected male littermates.

n = 4.

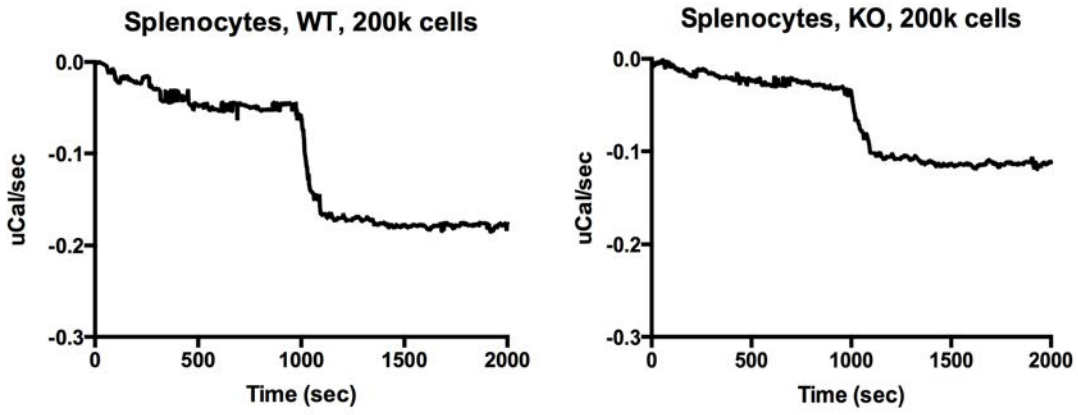
Figure 4.15



Chloroquine reduces heat production by splenocytes. ITC measurements comparing isolated splenocytes from control and CQ injected (3.5 mg/kg, IP) mice. Ctrl: control saline injected male littermates; CQ: chloroquine injected male littermates.

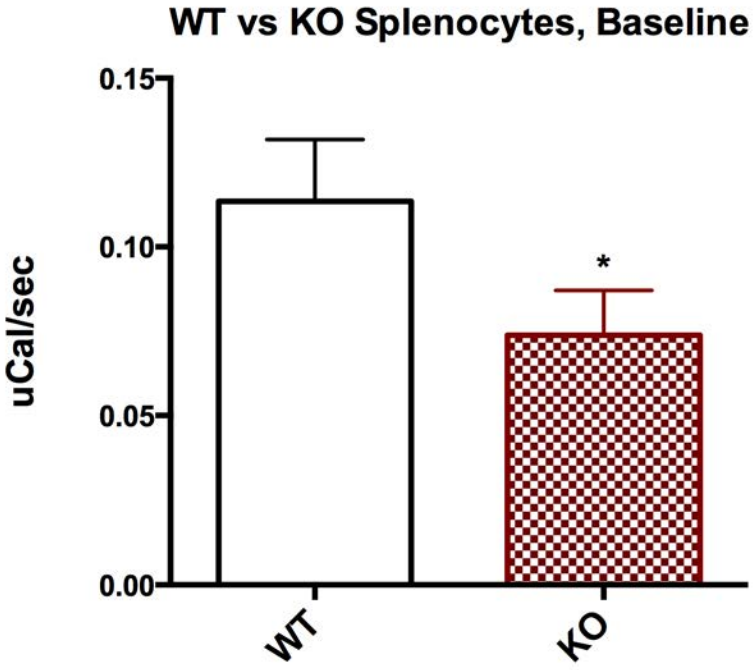
n = 5.

Figure 4.16



LIPA deficient splenocytes produce less heat than wildtype counterparts. Isolated splenocytes comparing wildtype and LIPA deficient littermates. Left: splenocytes from wildtype male littermates; Right: splenocytes from knockout male littermates.

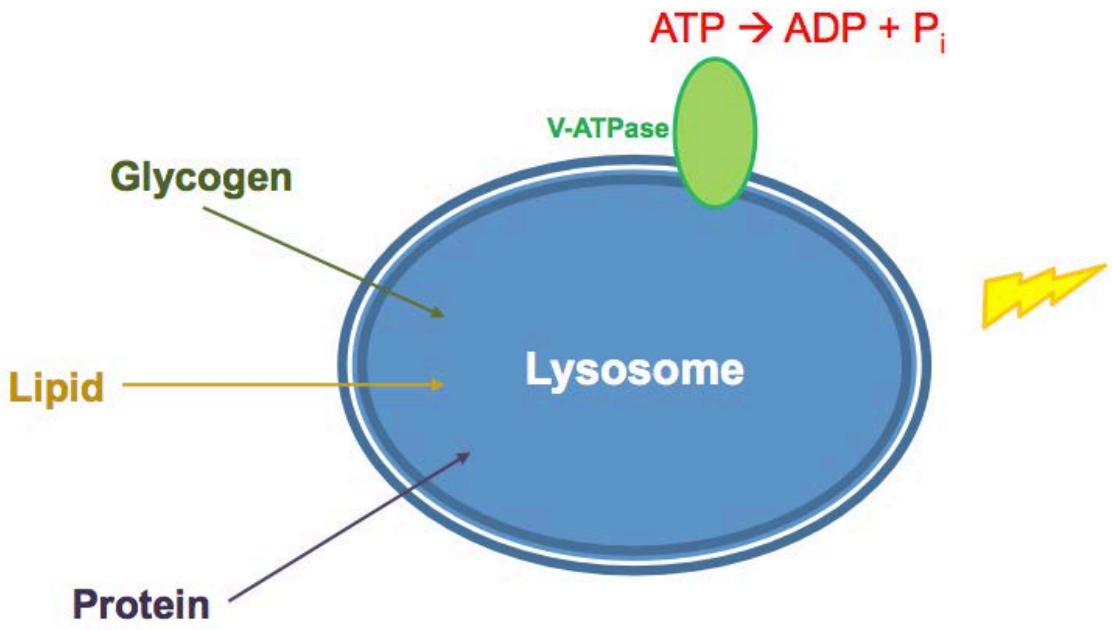
Figure 4.17



Heat production by wildtype and LIPA deficient splenocytes. ITC measurements of isolated splenocytes from wildtype and LIPA deficient mice. WT: wildtype male littermates; KO: LIPA deficient male littermates.

n = 4.

Figure 4.18



Summary model. Lysosomes are thermogenic organelles that contribute to overall body heat production through catabolic reactions, hydrolysis of ATP, and potentially an unidentified uncoupling mechanism.

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CHAPTER FIVE

LYSOSOMES ARE SUBSTRATE PROVIDERS OF NONSHIVERING THERMOGENESIS

Introduction

In the classic models of NST, cold exposure induces catecholamine release from efferent neurons, activating β3-adrenergic receptors on brown and beige adipocytes. This activation results in increased uncoupled mitochondrial oxidation (Tseng, Cypess, and Kahn 2010). Despite our understanding of the activation mechanisms of thermogenesis, questions regarding the cellular physiology of NST in the BAT still remain. In particular, free fatty acids (FFAs) are presumed to be key substrates for NST (Moriya and Hiroshige 1981; Vincent-Falquet et al. 1972; Wu et al. 2006; Guerra et al. 1998). In addition, it has also been assumed that free fatty acids used in uncoupled oxidation and thermogenesis are derived from neutral lipase-dependent lipolysis of lipid droplets from both white and thermogenic adipocytes. In this model, free fatty acids (FFA), are stored as triglycerides (TG) in brown, beige, and white adipocytes. These NST substrates are mobilized through action of lipid droplet-associated lipases without a role for lysosomal acid lipase (Duncan et al. 2007; Cannon and Nedergaard 2004; Virtanen 2014).

In response to catecholamine activation, FFAs derived from the white adipose tissue (WAT) TG are released into the circulation and taken up by the BAT; while FFAs from intracellular lipid droplet stores of brown and beige adipocytes are also thought to provide intracellular substrates for uncoupled oxidation.

Current models posit that TG lipid droplets in these adipocytes are mobilized through actions of the neutral lipase, including the critical adipose triglyceride lipase (ATGL/PNPLA2) (Duncan et al. 2007; Cannon and Nedergaard 2004; Virtanen 2014).

The cold intolerant phenotype of our LIPA deficient model suggests that LIPA may also provide FFAs to the mitochondria during cold challenge. To estimate the contribution, if any, of LIPA to lipolysis in thermoregulation at 4˚C, we compared LIPA deficiency with other lipase deficiencies. The data presented in this chapter provide insights to the extent of LIPA importance in nonshivering thermogenesis.

Hypothesis and Proposed Model

Lysosomal acid lipase is upregulated during cold exposure in order to provide the FFA substrates necessary for maintaining mitochondrial NST.

Methods and Materials

Quantitative Real-time Polymerase Chain Reaction.

Tissue samples were immediately frozen by liquid nitrogen from euthanized mice at the termination of experiments. RNA was extracted from frozen tissue samples using an acid-phenol reagent according to manufacturer's instructions (RNA-solv reagent, Omega Bio-tek). RNA was further purified by silica-membrane method from the manufacturer (E.Z.N.A. Total RNA Kit II, Omega Bio-tek) and then used as template for cDNA synthesis (qScript cDNA SuperMix, Quanta Biosciences). Quantitative real time PCR (qRT), was performed using white 384 well plates in the Roche LightCycler 480 machine. Analysis was done by normalizing expression to Rps3 and expressed in relative units using the DDCT-2 method, taking account of each primer efficiency calculated per in-run standard curve. Primer sequences are listed in Table 1.

Lysosomal acid lipase activity assay.

LIPA activity was determined using a 4-methylumbelliferyl oleate assay with 4-methyumbelliferone serving as the standard curve. 4-MUO was dissolved in hexane at 100 mg/ml and diluted 1 to 250 in 4% Triton X-100. Tissue extracts were prepared in the same way as western blot samples (see Immunoblots). 25 ul of diluted tissue extracts, 50 ul of diluted 4-MUO substrate, and 125 ul of assay buffer (0.2 M NaOAc and 0.01% Tween 80, pH 5) were incubated at 37flC for 30 minutes. To stop the reaction, 100 ul of 0.75 M Tris, pH 8.0 was added to each sample. Fluorescence was detected using (look up machine) at excitation 360 nm and emission 460 nm. Relative fluorescence units from each sample were then compared to that of the standard curve and normalized to wildtype controls.

Mice.

Ucp1 Cre mice were obtained commercially from Jackson Labs. Atgl fl/fl mice were provided from Dr. Erin Kershaw (University of Pittsburgh). Pompe mice were gifted by Jeffrey Pessin (Albert Einstein College of Medicine).

Cold challenge.

Mice were housed individually for up to 72 hours in 4-8flC. Cold challenge experiments for this chapter allowed ad libitum access to regular chow diet and water. Mice followed the same 12 hr day/12 hr night cycle of the Columbia ICM specific pathogen free housing.

Statistics.

Significance calculations of comparisons between two groups were determined by student's t-test. Significance calculations of multiple group comparisons were determined first by ANOVA and then Bonferroni correction. Key for all significance indications: * = p-value ≤ 0.05; ** = p-value ≤ 0.01; *** = p-value ≤ 0.001.

Figures represent one experiment out of four. Data are not combined to produce the figures or statistics. Error bars represent the standard deviation.

Results

Pompe mice are cold tolerant and increase LIPA activity upon cold exposure

Our data suggest that lysosomal function is necessary for the full thermogenic response of mice to a cold challenge. We chose to study LIPA-deficient animals, because lipid catabolism has been implicated in thermogenesis previously and LIPA expression and activity are induced in by cold challenge in BAT and other tissues. To test whether lysosome dysfunction generally impairs thermogenesis we studied a mouse model of Pompe Disease. Pompe mice lack acid alpha-glucosidase (GAA), an enzyme necessary for the breakdown of glycogen in the lysosome. As a reflection of the importance of glycogen in muscle, Pompe mice exhibit severe muscle pathology, including weakness and atrophy (Bijvoet et al. 1998; Vellodi 2005). Although effects of GAA deficiency in adipose tissue have not been well studied, we did not note any change in Gaa expression in muscle or adipose tissue following a cold challenge.

Pompe mice were cold challenged compared with age and sex matched littermate control mice. Temperatures were monitored every two hours for 12 hours at 4˚C using a rectal probe thermometer (Fig 5.1). We found no differences in body temperature in Pompe compared to wildtype controls, demonstrating that impairment of lysosome function per se is not sufficient to compromise thermogenesis.

Intriguingly, while the Pompe mice were cold tolerant, we found an increase in LIPA activity in BAT at 4C (Fig 5.2). We found no changes in other lipases (data not shown).

Ucp1 Cre, Atgl fl/fl mice are cold tolerant.

Two groups (Zechner and Sul) reported that deficiency of ATGL/PNPLA2 causes cold intolerance (Haemmerle et al. 2006; Ahmadian et al. 2011). Mice with whole body deficiency were found to die within hours of being placed at 4˚C. However, subsequent studies have shown that cardiac specific *Atgl* expression in otherwise deficient mice prevents the cold intolerance. These data argue that neutral lipolysis of TG from fat is not necessary for thermoregulation. The cause of death of mice completely deficient in Atgl is thought to be secondary to the hearts of these mice being unable to tolerate the high sympathetic tone of a cold challenge. The Sul group, however, reported that Atgl deletion driven by the Fabp4/aP2 promoter leads to modest cold intolerance. Although the goal was to direct deletion in adipocytes, Fabp4 is expressed in other cells as well including macrophages and some areas of the brain. To address the question of whether neutral lipolysis in adipocytes is required for NST, we used an Adiponectin-Cre line of mice to provide specific deletion in all adipocytes and a Ucp1-Cre line of mice to specifically delete Atgl in thermogenic adipocytes. Mice lacking with impaired neutral lipolysis in adipocytes (Fig 5.3) or in brown/beige adipocytes (Fig 5.4) were cold tolerant and had no difference in body temperature compared to littermate control mice.

Ucp1 Cre, Atgl fl/fl mice show induction of lysosomal program in the brown adipose tissue compared to wildtype littermate controls.

While we established that ATGL is not necessary for thermogenesis in brown and beige adipocytes, we sought to determine whether deletion caused any compensatory changes in other lipases. Using qRT-PCR, we measured expression of the genes, *Lipa*, *Hsl*, and *Mgl* (Fig 5.5). These data demonstrate that *Lipa*,

Hsl, but not *Mgl* mRNA are upregulated in response to the ablation of *Atgl*, suggesting that other lipases are likely upregulated in response to the lack of ATGL in the tissue.

Because our interest is primarily with the lysosome, we further validated the importance of various lysosomal genes in Ucp1 Cre, Atgl fl/fl mice. QRT-PCR showed that several lysosomal gene transcripts are upregulated during cold challenge in Ucp1-Cre; Atgl fl/fl mice (Fig 5.6). LIPA activity was also augmented under these conditions (Fig 5.7). These data show that lysosomes are increased when ATGL function is disrupted in thermogenic tissues.

Proposed summary model (Fig 5.8).

Cold tolerance of Pompe mice indicate that glycogen is not necessary for thermogenic response and supports the specificity of LIPA activity during cold challenge. Data presented in this chapter demonstrate the primary neutral lipase, ATGL, is dispensable for NST. However, the increase in expression of other lipase suggests that there is compensation and imply a contribution for ATGL in generating substrates for NST. The upregulation in expression of *Lipa* also provides indirect evidence that LIPA has a role in providing FFAs for NST, either by activating UCP1 or providing substrate for uncoupled oxidative metabolism.

Conclusions

Based on the data from this chapter, we see potential evidence supporting the contribution of LIPA in contributing substrates needed for nonshivering thermogenesis. Published data on HSL and data in this chapter on the Ucp1 Cre, Atgl fl/fl indicate that these neutral lipases are not necessary in BAT during cold challenge contrary to current dogma. Moreover, LIPA activity and the lysosomal program in brown adipose tissue is induced in the floxed mice compared to their wildtype littermate controls.

One of the main concerns regarding the LIPA mouse model is its nonspecific effects on lysosome function overall. When we examined the cold tolerance of another lysosomal storage disease model however, the Pompe mouse, we found that those mice were able to survive cold challenge and also increased LIPA activity upon cold exposure. Without substrates from food intake, the severity of cold intolerance in the LIPA KO mice was further exacerbated. Taken together, these data suggest a particular specific importance of LIPA in cold challenge that may be a result of its function in producing FFAs from lipids.

Further hints supporting the substrate hypothesis model come from the effect of nutritional status on cold survivability outcomes. Our various feeding patterns showed that LIPA deficient mice needed to be fed at the start of cold challenge to survive (data not shown). We hypothesize that the feeding may alleviate the substrate load on the lysosome, allowing for other substrate sources to be used by the BAT. This conjecture would rationalize how fasting prior to cold challenge would increase lysosomal activity, in particular, LIPA activity during cold exposure. Additionally, in Chapter three we found that fasting is required for a lysosomal program after pharmacological induction of NST by CL-316,243. While

these data do not definitely show that LIPA is necessary in providing substrates, they point to the importance of substrate provision with respect to lysosomes during cold challenge.

Limitations and Concerns

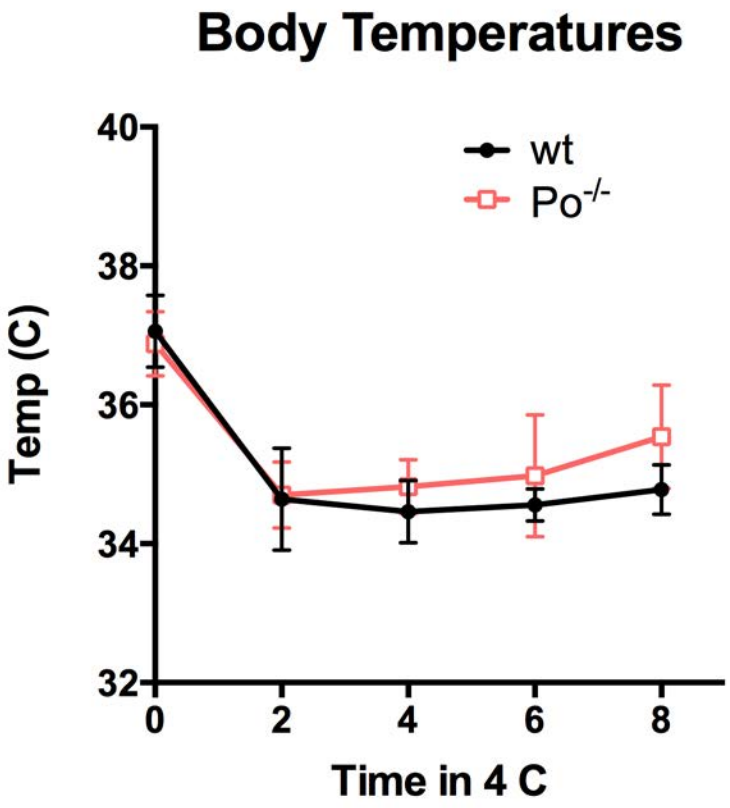
Indirect data.

The majority of the data presented in this chapter are indirect and require assumptions to interpret. For instance, our Ucp1 Cre, Atgl fl/fl model genetically ablates *Atgl* function in UCP1 expressing tissues, but does not take account of free fatty acid species from other sources. As current research studies, as well as this dissertation, have validated the importance of neutral lipase action in white adipose tissue (WAT), it is possible that WAT-derived FFAs compensating for the ATGL inadequacy in BAT of the Ucp1 Cre, Atgl fl/fl mice. Further, while we did notice an increase of LIPA activity in both the Ucp1 Cre, Atgl fl/fl mice and the Pompe mice, it is not clear how much contribution LIPA is making to the overall FFA pool used by the mitochondria during cold challenge.

Our fasting and refeeding studies show a clear effect on survivability in LIPA mice. However, this merely supports, but not prove, the substrate provision model. The chow diet fed to our mice is approximately 2-3% fat; by far, the majority of the energy consumed by the mice is in the form of glucose and carbohydrates. Glucose is important in muscle and somewhat in BAT. However, heat generation from shivering is the critical in cold survivability in the first few hours of a cold challenge. By not measuring the shivering activity in our mice, it is unclear how much of our effects are due to a muscle, not BAT phenotype.

Figures

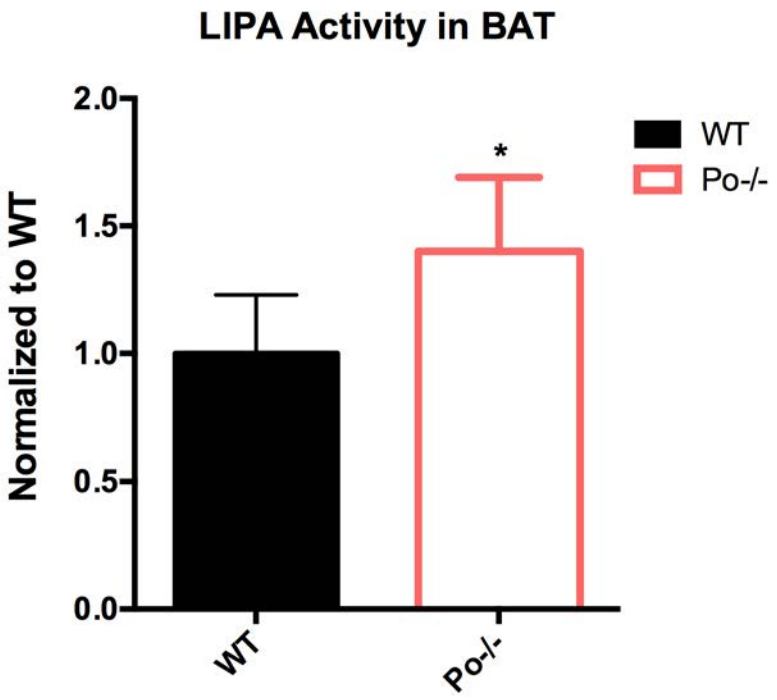
Figure 5.1: Pompe mice are cold tolerant.



Pompe mice are cold tolerant. Temperature data of cold challenged Pompe mice and wildtype controls. Po-/-: Pompe.

n = 6.

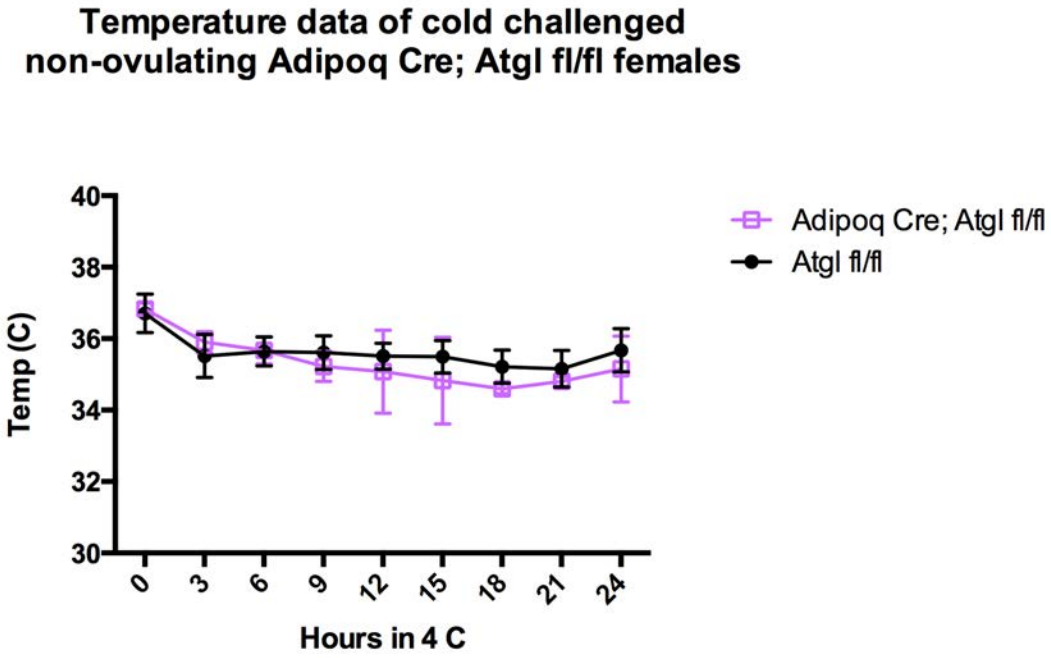
Figure 5.2: LIPA activity is increased in BAT of Pompe mice.



LIPA activity is increased in BAT of Pompe mice. LIPA activity in BAT of Pompe mice compared to wildtype controls, as measured by the 4-MUO assay. Po-/-: Pompe

n = 6

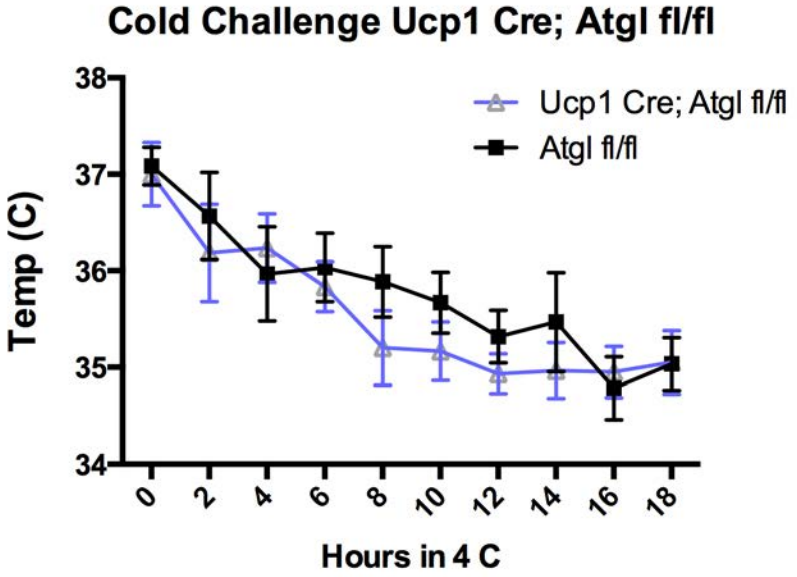
Figure 5.3: Non-ovulating Adipoq Cre, Atgl fl/fl females are cold tolerant



Cold challenge of non-ovulating Adipoq Cre; Atgl fl/fl mice. Rectal temperature data from cold challenged Adipoq Cre; Atgl fl/fl nonovulating females and littermate controls.

n = 4

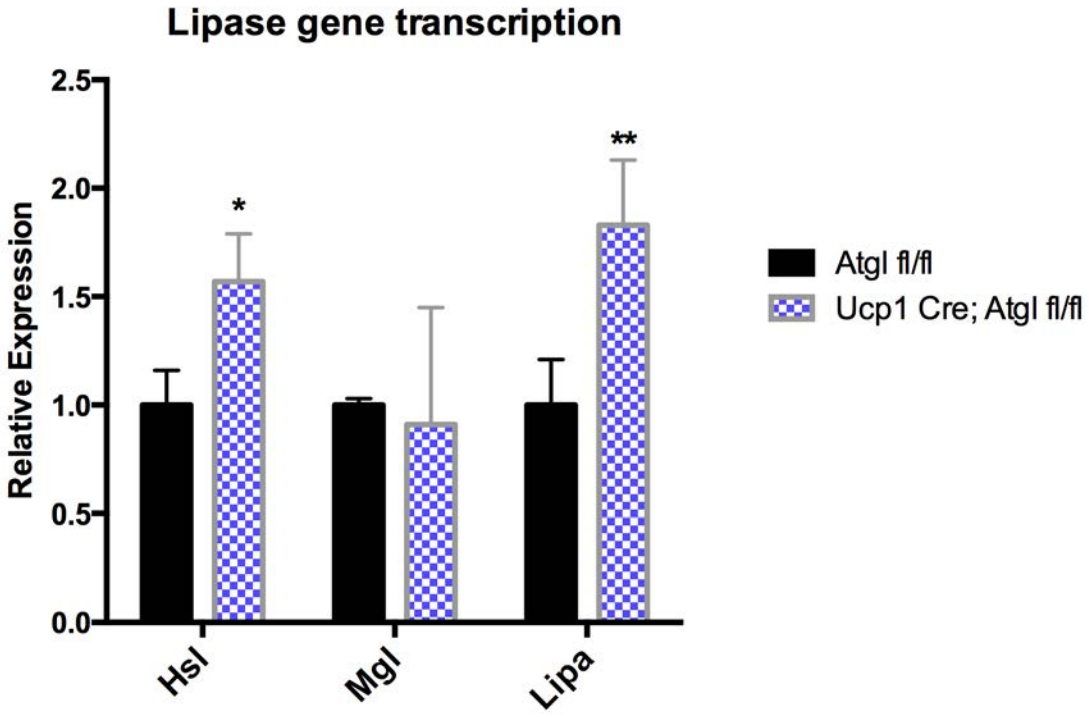
Figure 5.4: Ucp1 Cre, Atgl fl/fl mice are cold tolerant



Ucp1 Cre, Atgl fl/fl are cold tolerant. Temperature data of Atgl fl/fl and Ucp1 Cre, Atgl fl/fl littermates in 4 C.

n = 6

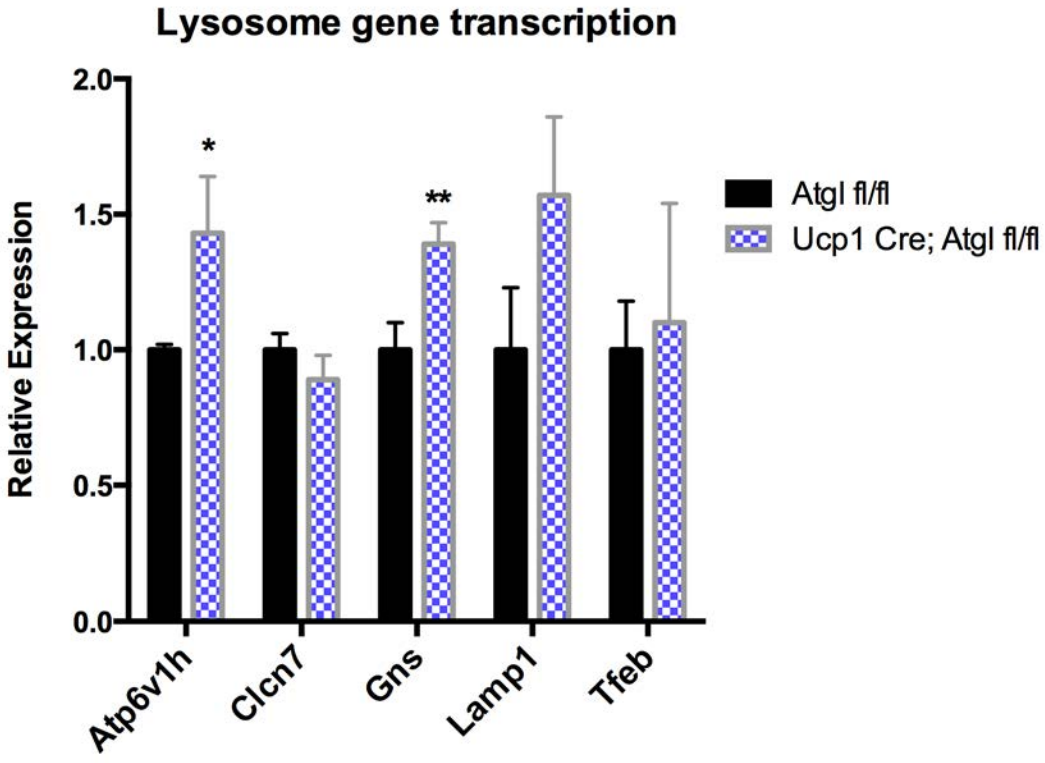
Figure 5.5: Cold challenged Ucp1 Cre, Atgl fl/fl increase transcription of *Hsl* and *Lipa*



Cold challenged Ucp1 Cre; Atgl fl/fl mice show increases in gene transcription of certain lipases. Quantitative real time PCR of *Hsl*, *Mgl*, and *Lipa* of whole BAT depots from Atgl fl/fl and Ucp1 Cre; Atgl fl/fl male littermates.

n = 6

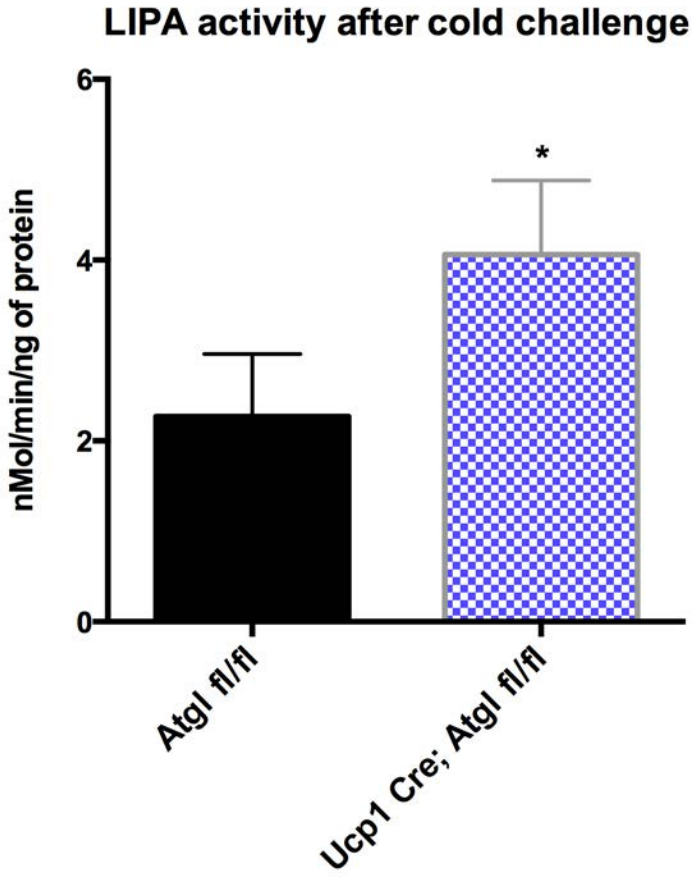
Figure 5.6: Cold challenge increases transcription of certain lysosomal genes in BAT of Ucp1 Cre; Atgl fl/fl mice.



Cold challenge increases transcription of certain lysosomal genes in BAT of Ucp1 Cre; Atgl fl/fl mice.
Quantitative real-time PCR of *Atp6v1h*, *Clcn7*, *Gns*, *Lamp1*, and *Tfeb* in isolated BAT depots of cold challenged Atgl fl/fl and Ucp1 Cre; Atgl fl/fl male littermates.

n = 6

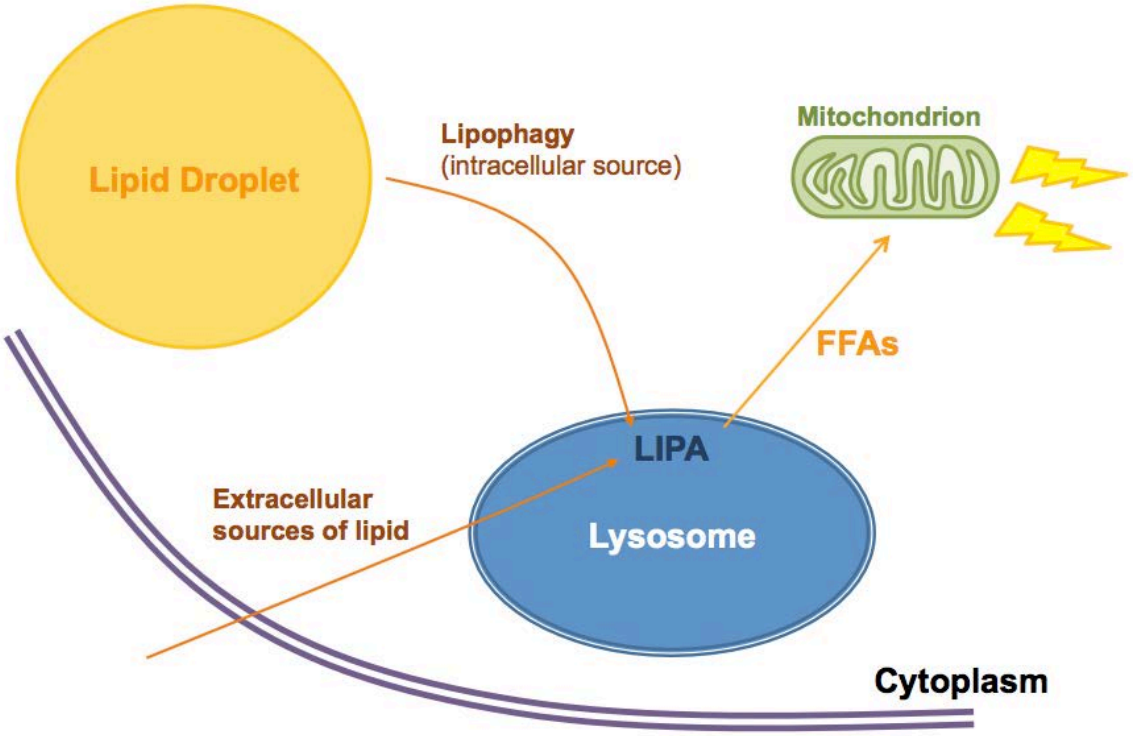
Figure 5.7: LIPA activity increases in cold challenged Ucp1 Cre, Atgl fl/fl mice



Increased LIPA activity in Atgl deficient BAT after cold challenge. LIPA activity as measured by the 4-MUO assay comparing whole BAT from Atgl fl/fl and Ucp1 Cre; Atgl fl/fl male littermates.

n = 6

Figure 5.8: Proposed Summary Model



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CHAPTER SIX

CONCLUSIONS, FUTURE DIRECTIONS, AND DISCUSSION

Conclusions

Lysosomes play critical roles in immune responses, protection from degenerative diseases and aging, and metabolic function of cells. Our studies here have uncovered a previously unappreciated role for lysosomes in the generation of heat. When challenged by a drop in ambient temperature or by pro-febrile stimuli, mammals activate thermogenesis to either maintain or increase body temperature. We initially observed that in response to these thermogenic stimuli, mice increase lysosomal programs in multiple tissue. Even fly larvae activate a lysosomal transcriptional program in response to a cold challenge. Disruption of lysosome function either pharmacologically via chloroquine administration or from ablation of lysosomal acid lipase reduces body temperature during a cold challenge and fever induction. These findings strongly implicate lysosomes in the generation of heat and body temperature regulation.

To study the function of lysosomes in regulated thermogenesis, we employed pharmacologic and genetic approaches, uncovering a complex set of responses to impaired lysosomal function. Inhibition by both means lowered body temperature and induced varying degrees of compensatory increase in the canonical UCP1 thermogenic program. Chloroquine impairment in lysosome function led to an increase in UCP1 in both subcutaneous and brown adipose tissue depots, suggesting a compensatory attempt to increase thermogenesis via the classical pathway. In *Lipa-/-* mice, there too was an increase in the canonical pathway in subcutaneous adipose tissue, again as an apparent compensatory mechanism.

Consistent with a compensatory effect, upstream signaling as measured by MAP kinase phosphorylation was increased. However, in BAT LIPA deficiency, the increase was associated with a modest reduction in the expression of canonical thermogenic genes, including a 35% reduction in *Ucp1.* We conclude that while lysosome function is not required for uncoupled thermogenesis program, severe impairments in lysosomal lipid catabolism likely reduce the signal required for the full program.

The modest and inconsistent effect of lysosome impairment on canonical thermogenic program across tissues argued that lysosomes contributed to thermogenesis via a different, non-classical mechanism. Given the thermogenic reality that degradation of biomolecules with high energy bonds requires release of energy, it seems necessary that lysosomes must contribute directly to thermogenesis. In addition, lysosomes, like mitochondria and sarcoplasmic recticula, generate an ion gradient (H^+) in a energy consuming process. Approximately 50-60% of the energy released during the hydrolysis of ATP to ADP via the V-ATPase pump is in the form of heat. Further, it is possible that this acidification process could be uncoupled in a manner similar to what occurs in mitochondria and across the sarcoplasmic recticula in other well elucidated forms of NST.

Testing this hypothesis requires direct measurement of heat generation by lysosomes. We attempted to identify methods to do this and found isothermal titration calorimetry (ITC) to be a powerful tool. We established ITC as a reliable method to measure heat generation by cells, and as a first step we measured heat production from cells in which lysosome function was disrupted. BAT cells as well as nonUCP1 expressing splenocytes generate heat that is in part dependent upon lysosome function. These data indicate that the reduction in

thermogenesis caused by CQ or LIPA deficiency is not limited to brown, beige adipocytes, or myocytes. With further development, we hope to be able to measure the heat generated by isolated lysosomes.

The traditional model of thermogenesis requires FFA mobilization from WAT and BAT sources via the action of neutral lipases, particularly ATGL. However, contrary to some published studies using the leaky adipocyte protein 2 (Ap2) Cre to drive *Atgl* disruption in tissues, our Ucp1 Cre, Atgl fl/fl mice showed normal thermogenic response to cold exposure. Instead, in these mice, we found an increase in LIPA activity. These data support the substrate provision model of LIPA. We propose that during cold challenge, LIPA acts as one of the main lipases that provide the substrates necessary for maintain the FFA pool for thermogenesis.

Our work identifies lysosomes as critical for body temperature homeostasis in mice. These studies add a new dimension to the understanding of lysosomes and their role in cell and systemic physiology. The mechanisms by which lysosomes contribute to regulated thermogenesis still need to be further delineate. However, we can make several conclusions: (1) Normal lysosome function is required for full thermal responses to cold or febrile stimuli and the alterations in heat generation; (2) Lysosomes contribute to thermogenesis of BAT and splenic cells.

Additional Future Directions

Chapter Two: Heat production and lysosomes.

In Chapter Two, we demonstrated in mice that lysosomes are induced in response to cold and LPS/poly(I:C) and that impairment of lysosomal function reduces body temperature in mice in response to thermogenic stimuli. A more complete profile of the lysosomal program induced by these stimuli and the cells responsible for them will be critical for delineating the mechanisms that regulate lysosomal thermogenesis.

In our efforts to understand the regulation of lysosomes in response to thermogenic stimuli, we surveyed multiple mouse organs by qPCR and discovered increased expression of lysosomal genes in nonUCP1 tissues. An unbiased profile gene expression in these tissues (spleen, skeletal muscle, liver) would provide not only a more complete picture of the lysosomal program activated by cold and pathogen antigens, but may provide hints to the regulatory pathways upstream and any changes in the cell population of responsive tissues. Our data in spleen suggest that cold can alter the recruitment as well as the profile of cells in tissues such as the spleen.

Our poikilotherm data are derived solely from *Drosophila melanogaster* larvae in their third instar stage. However, our model, if accurate, would predict similar responses in other organisms. We did not test cold exposure at other stages or in other poikilotherms at significant extent. Cold exposure in *C. elegans* yielded some evidence supporting our *Drosophila* studies (data not shown). Further analyses verifying protein expression and activity in multiple poikilotherms would help in corroborating our observations. If, however, we can demonstrate that cold induces lysosomes in *Drosophila* larvae (or any other genetically tractable

organism) and we can measure a thermogenic phenotype (e.g. survival, developmental rate or temperature) then we would have the opportunity to perform screens in search of the genes involved in lysosome dependent thermogenesis.

Due to technical difficulties and limits in availability, we were not able to obtain UCP1 deficient mice. C57BL/6J *Ucp1 -/-* mice are known to be able to survive cold challenge after a three week acclimation in 18˚C, supposedly through shivering thermogenesis. These mice will represent an important opportunity to test whether lysosome thermogenesis is necessary for the cold survival in the absence of uncoupled oxidation. Potentially, our lysosome phenotype may be upregulated in the acclimated mice compared to mice raised in thermoneutrality. Examining lysosomal function in these mice would therefore reveal the extent of lysosome importance in thermoregulation outside of the traditional "classical nonshivering thermogenesis." A double mutant lacking UCP1 and normal lysosome function, e.g. *Lipa* deficiency, would allow us to understand the relative importance of lysosomes in thermoregulation.

While we tested hyperthermia in the form of pyrexia, other inductions were not explored. Increasing environmental temperature to determine if lysosomes are actively downregulated would provide additionally support our proposed mechanism.

Chapter Three: Lysosomes are upstream regulators of thermogenic genes.

Neither genetic ablation of the critical autophagy gene, *Atg7*, in adipose tissue nor in myf5+ lineage (brown adipocytes and muscle) disrupt cold tolerance

(Martinez-Lopez et al. 2013; Singh et al. 2009). Those observations suggest that autophagy is insufficient to explain the dependence of thermoregulation on lysosomal function, at least not in known thermogenic tissues. We are in the process of generating mice that lack *Atg7* in thermogenic adipocytes using *Ucp1-Cre and* Atg7 fl alleles. This would allow us to confirm that autophagy in BAT is not required for thermoregulation.

The discovery of TFEB and its critical role in transcriptional regulation of catabolism and lysosome biogenesis was published within the last decade (Sardiello et al. 2009). Hence, the study of lysosomes and their transcriptional regulation is still in its early stages. Our data, while not conclusive, suggest that a factor other than, or in addition to, TFEB is critical for cold-induced transcription of lysosomal genes. If, like TFEB, the cold dependent transcription factor resides in a complex at the lysosome membrane, then proteomic studies examining the lysosome membrane prior and post cold challenge may identify candidate TFs that are involved. One potential transcription factor family involved in autophagy and metabolism is FOXO (Zhao et al. 2008; Hariharan et al. 2010; Ning, Li, and Qiu 2015; Sengupta et al. 2009; Milan et al. 2015; Xu et al. 2011; Wang et al. 2016; van der Vos et al. 2012). Future studies should consider measure levels of the various FOXOs in BAT of both wildtype as well as LIPA KO mice prior and post cold exposure.

In *C. elegans* studies, MXL-3, (MAX ortholog) prevents HLH-30 (TFEB orthologue) transcriptional actions by functioning as a repressor for HLH-30 downstream target genes, particularly lipases. MAX itself is not affected in mammalian cells during starvation; other repressors and inhibitors, still unknown, are likely involved in the modulation of TFEB actions (O'Rourke and Gary

2013). While we struggled to measure MAX, additional studies in identifying and characterizing TFEB modulators may also explain our lysosomal-NST relationship.

Our data showed a reduction in PGC1α expression after cold challenge in our LIPA KO mice. Interestingly, PGC1α is crucial in muscle function (Oliveira et al. 2004; Leick et al. 2010) and shivering during cold exposure. The whole body deficiency and our technical limitations in measuring muscle activity prevented us from measuring the contribution of muscle shivering in our phenotype. The drop in body temperature in *Lipa-/-* mice may be explained by lysosomal effects on *Pgc1α* transcription in muscle. Future studies to measure muscle contraction as well as PGC1α expression may be necessary to discern the various contributions to body temperature regulation in other tissues.

Chapter Four: Lysosomes as thermogenic organelles.

Chapter four demonstrated and validated the utility of microcalorimetry in measuring thermogenesis of cells. We found that we could quantitatively measure heat generated by cells. We hope this proves to be a useful tool in studying thermogenesis and metabolism more generally. In the future, we hope to adapt the technique to measure the heat generated by organelles. While there will be technical challenges as well as hurdles to overcome in interpreting such data, we hope that it will provide direct measures of at least the thermogenic potential of lysosomes.

The measurements performed to date, however, have been of heat generated by whole cells isolated from whole tissues. This limits our ability to interpret these data in two ways: 1) Cellular heterogeneity prevents us from determining the relative contribution to thermogenesis of each cell type; 2) The direct contribution of lysosomes to thermogenesis can only be inferred by measuring the heat generated from cells with intact and impaired lysosomal function. Despite these limitations, we are excited that this technique will prove useful in other studies of thermogenesis, and we are hopeful that approaches will be developed to overcome these limitations.

One immediate use for this technology would be to study BAT from UCP1 deficient mice. Controversy exist as to whether UCP1-deficient BAT can actually generate heat. Cannon and Nedergaard maintain that in the absence of UCP1, BAT is not thermogenic and that mice maintain body temperature by shivering. The slow acclimation, in their estimate, permits them to develop shivering "endurance." Using the ITC techniques developed here, we could test the Cannon-Nedergaard hypothesis directly. BAT can be removed and isolated from thermoneutral acclimated UCP1 deficient mice and cold challenged, 18˚C acclimated UCP1 deficient mice. Heat production by explanted, isolated BAT will therefore differentiate the heat contribution of shivering thermogenesis from nonshivering thermogenesis with the ITC methodology established in this dissertation.

Chapter Five: Additional roles for the lysosome during thermogenic stimuli.

The final data chapter in this dissertation presents a number of limitations. LIPA activity was measured only in Ucp1 Cre, Atgl fl/fl and Pompe mice. While cold

challenges were performed on the Pompe mouse, more LSD models should be examined. The Pompe mouse primarily affects skeletal muscle and therefore the reason Pompe mice are not cold intolerant in the way that *Lipa-/-* mice are may simply reflect that mitochondria in key thermogenic tissues are not significantly impaired, nor appropriate for testing lysosome importance in BAT. Other potential LSDs to examine include Niemann Pick disease type C1 (NPC1) disruption, Danon, or Gaucher. Using these other LSD models will provide a better understanding of the effects of lipids or overall lysosome disruption on nonshivering thermogenesis.

However, ideally LIPA activity should be assessed in Ucp1 Cre, Hsl fl/fl and other models of lysosomal storage disease. Compensatory mechanisms of these models also add confounding factors that complicate our model. We would use lipid tracing and labeling techniques in the diet during cold challenge to understand the fate of lipids. Labeled lipids detected in the lysosome during cold challenge versus other potential lipid compartments would suggest preferential shuttling of lipids to LIPA versus neutral lipases.

The proposed substrate provision model depends on lysosomes providing lipids to the mitochondria. As discussed in the previous section, the mechanism responsible for free fatty acid delivery to the michondrion is unknown. Disruption of fatty acid oxidation in the mitochondria through carnitine palmitoyltransferase I (CPT1) would uncouple fatty acids in mitochondria from its delivery mechanism. Further examination of the pathways altered, in particular, verification of lysosomal alteration, would add to the understanding of LIPA-derived free fatty acids in NST.

Discussion

UCP1 independent nonshivering thermogenesis

Our studies suggest that lysosome catabolism serves as a form nonshivering thermogenesis. As noted below, some contend that UCP1 independent thermogenesis does not exist (Cannon and Nedergaard 2011; Golozoubova et al. 2001).

"Nonshivering thermogenesis is fully due to brown adipose tissue activity; adaptation corresponds to the recruitment of this tissue...Although all mammals respond to injected/infused norepinephrine (noradrenaline) with an increase in metabolism, in non-adapted mammals this increase mainly represents the response of organs not involved in nonshivering thermogenesis; only the increase after adaptation represents nonshivering thermogenesis." (Cannon and Nedergaard, 2011)

This position reflects the interpretation of data from older studies stating that measured NST capacity during thermoneutrality after catecholamine administration is attributable entirely to UCP1 dependent thermogenesis. However, if NST is limited to brown adipose tissue activity and activation of UCP1, then the ability for *Ucp1* ablated mice to survive in colder temperatures without increasing shivering from initial exposure suggests that other factors may be at play (Golozoubova et al. 2001). In the original paper, Golozoubova, et. al., acclimated wildtype and UCP1-deficient mice for three weeks at thermoneutrality, room temperature, and 18 C. When acclimated to 18˚C, the *Ucp1* ablated mice were able to survive in the cold. The authors claim that this consistency in shivering at the start and end of acclimation is proof that no other

alternative nonshivering thermogenic pathway could exist. The difference in survival with and without cold acclimation is attributed to muscle endurance. However, during this acclimation, lysosomal activity may have gradually increased, an observation that may not be captured without *a priori* knowledge. As discussed in the "Future Directions" section of this chapter, UCP1 deficient mice should be tested for lysosomal activity prior and post 18˚C acclimation.

Ultimately, the classical "nonshivering thermogenesis" defined by Nedegaard, Cannon, and other experts in the field may be too limited. Other known mechanisms that can increase body temperature such as vasoconstriction and piloerection are not considered as part of their definition of NST response (Cannon and Nedergaard 2011). Without *a priori* understanding of lysosomal function in NST, our characterization of lysosomes may have been lost in earlier studies. This dissertation asserts that lysosomes, whose activity is modifiable (to an extent), should be considered as part of the NST definition.

Thermoneutrality and acclimation.

One of the main lessons learned from Golozoubova, et. al, and other studies from both Nedergaard and Cannon is the effect of acclimation on NST research. Our experiments were performed on mice that were raised in an ambient temperature of 20-25˚C. Assuming that this acclimation has no effect on lysosome activity and is purely increasing muscle endurance, then our LIPA KO mice may simply suffer from poor muscle endurance (See "Future Experiments."). However, LIPA deficient mice, if fed, exhibit a U-shaped temperature curve. Their body temperatures never fully recover, suggesting that shivering alone may not describe our cold intolerance phenotype. While these data do not definitively rule

out shivering as the sole factor in determining cold survivability of our LIPA deficient mice, they do raise doubts in the applicability of the counterargument.

Measuring nonshivering thermogenesis.

Nedergaard and Cannon have long argued that a cold challenge does not permit accurate measurement of NST. Instead, they insist cold challenges primarily reflect shivering and often failure to maintain body temperature is a measure of exhaustion. Catecholamine injection is the gold standard for inducing BAT and measuring its capacity; however, this approach has a myriad of limitations. First, as with all pharmacological interventions, catecholamines have a number of targets beyond thermogenic adipocytes. Even CL-316,243, a β3 adrenergic receptor-specific agonist activates β3-adrenergic receptors expressed on the bladder (Cypess et al. 2015), gallbladder (Guillaume et al. 1994), and skeletal muscle (Chamberlain et al. 1999). Effects on these tissues would therefore be expected to overestimate NST attributable to BAT.

Second, catecholamine administration is recommended to be done at thermoneutrality to avoid sympathetic activation at lower temperatures (Cannon and Nedergaard 2011); however, even thermoneutral conditions can activate UCP1 through diet (Hervey and G. 1981). Third, injections often increases stress, which alone raises body temperature (through endogenous catecholamines) and various other physiological and confounding responses. Lastly, the biggest limitation in catecholamine injection to measure NST is that it assumes that NST can only be activated by adrenergic means. Our data show that CL-316,243 administration alone is incapable of increasing the lysosomal transcription we discovered in the BAT. This same program however, is activated by cold

exposure alone. Norepinephrine may be additionally needed (see "Future Experiments'), as it also activates β1-adrenergic receptor, contributing to NST activation. Unfortunately, this agonist targets a myriad of tissues outside of BAT. Nonetheless, fasting combined with β3-adrenergic receptor stimulation was required to simulate a similar lysosomal response to cold challenge, suggesting that NST may not be equated to adrenergic thermogenesis.

Therefore, the study of NST is limited by the available tools; neither cold exposure nor catecholamine activation can accurately describe nor measure NST capacity and activity in animals. Chapter four of this dissertation proposes an *ex vivo* method of measuring heat production via ITC or IMC instruments. If combined with other measurements, the microcalorimetry approach may allow for more accurate study of NST.

Febrile Response.

While Cannon and Nedergaard argue that uncoupled oxidation is the only source of nonshivering thermogenesis in response to a cold challenge, it is clear that heat production can occur in the absence of UCP1 and mitochondrial uncoupling. Although "shivering" and alterations in blood flow can contribute to the development of fever, hypermetabolism in the absence of shivering or shaking exists. Given that hypermetabolic febrile responses occur equally in individuals with little brown adipose tissue (obese and elderly), in the absence UCP1 and shivering (Himms-Hagen and Jean 2009; Gabaldón, McDonald, and Horwitz 1998; Cypess and Kahn 2010; Okamatsu-Ogura et al. 2007; Lenhardt et al. 1998), there likely are nonshivering forms of thermogenesis. We have argued that lysosomes play a role in thermogenesis of fever. While critics may argue that our

pharmacologic and genetic manipulations impair cold response because of reduced muscle function and endurance, the argument is less compelling in the context of febrile response experiments.

Physiological responses to hyperthermia.

While we have framed our study of lysosome catabolism and thermogenesis in the context of increasing thermogenesis, our model predicts that at higher ambient temperatures or excessive heat generation, lysosome function may be downregulated to reduce lysosome-derived heat. This can be tested by placing animals at multiple temperatures and demonstrating an inverse relationship between higher ambient temperatures and lower lysosome activity. It may be possible that different tissues may alter their lysosomal program through a range of temperatures. In this way, lysosomal activity may not only increase during a cold challenge, but also decrease during excess heat.

Brown vs. Beige Adipocytes

Data in Chapters two and three suggest that lysosomal thermogenesis differentiates brown from beige adipocytes. We find that lysosomal function is induced during a cold challenge and important in brown adipose tissue, whereas activation of lysosomal thermogenesis appears to be absent from beige cell containing subcutaneous adipose tissue. Previous studies have established differences in lineage and transcriptional activation between the two cells (Jun Wu et al. 2012; J. Wu et al., n.d.). Our data add to another functional difference that distinguishes brown from beige adipocytes.

Other thermogenic pathways.

The regulation of brown and beige adipocyte thermogenesis in mammals depends upon a complex neuroendocrine system in which peripheral sensing of

temperature are relayed to key nuclei in the hypothalamus that subsequently activate autonomic and endocrine signals to modulate thermogenesis. The targets for this neuroendocrine regulation include the control of the UCP1 program in adipocytes and circulatory responses to maintain temperature homeostasis (Fan et al. 2005; Morrison, Madden, and Domenico 2014; Morrison, Kazuhiro, and Madden 2008; Guernsey, D.L., and I.S. 1983). Interestingly, while *Drosophila* is not a homeotherm and not known to possess an integrate temperature regulatory system, we found that, similar to our studies in mice, a cold challenge induces a lysosomal transcriptional program. In addition to arguing that the lysosome's contribution to thermogenesis is evolutionarily ancient, it also suggests that it is activated by a system that is different than that which regulates UCP1 thermogenesis. Others have characterized the "cold shock" response of *Drosophila* demonstrating that at least at the cellular level they respond to cold temperature by activating the transcription of proteins that serve to protect the fly (Czajka and Lee 1988; Ayar et al. 2010; Moskalev et al. 2015; Burton et al. 1988; Goto 2001). Consistent with our data, fasting, which induces catabolism and lysosomal function, increases cold tolerance in *Drosophila* (Le Bourg 2015). Recent studies from the Spiegelman laboratory found evidence for a cell autonomous system of temperature sense in mammalian cells as well, specifically that Myf5 negative, or inducible, adipocytes are capable of sensing temperature directly, without the need to activate its β3-adrenergic receptor (Ye et al. 2013). Identification the stimuli to cold-induced lysosome biogenesis will help us establish whether there are cell autonomous mechanism that contribute to thermogenesis.

Lysosomes and central control of body temperature regulation.

Work from the Singh lab has identified autophagy in Proopiomelanocortin (POMC) neurons (important in regulating anorectic behavior) in the central

regulation of temperature response via effects on BAT and the liver (Martinez-Lopez et al. 2016). Cold exposure increases autophagy in POMC neurons, leading to the activation of lipophagy in BAT. Additionally, Martinez-Lopez et. al., indicate that this autophagy induction is necessary for lipid mobilization in BAT, as the denervation of BAT blocks lipid utilization. Our LIPA deficient mouse model may have impaired autophagy in the hypothalamus, which based on these data could reduce lipid mobilization in BAT. However, the deletion of *Atg7* in hypothalamic POMC neurons does *not* render mice cold intolerant, and we find evidence of increased, not reduced, sympathetic signaling in BAT. Furthermore, fat-specific deletion of *Atg7* does not render mice cold-intolerant. LIPA, therefore, has a more central role in thermogenesis. Its phenotype does not appear to be attributable to alterations in autophagy, either centrally or peripherally.

Lysosome regulatory pathways.

As a multifunctional organelle, the lysosome must integrate its functions with other cellular systems and processes, including phagocytosis, autophagy, and program cell death (Settembre et al. 2013; Ballabio 2016). Currently, only one protein, TFEB, has been shown be a master regulator of lysosome biogenesis and function. Not surprisingly, TFEB is also an important transcriptional regulator of autophagy. In our attempt to identify the regulatory pathway that activates lysosome transcription in response to cold, we did not find convincing evidence that TFEB or related proteins, TFE3/MITF, played a role. Nor could we find evidence for the regulation of the negative regulator of TFEB, MAX in our experimental conditions. There are several strategies that can be employed to identify key regulatory molecules: 1) Identify transcriptional regulators by analyzing complexes associated with the *Lipa* promoter in BAT upon cold challenge; 2) Set up a screen in *Drosophila* to identify genes that prevent cold-induced lysosome biogenesis; 3) Perform proteomic analysis of lysosomes isolated

from mice a room temperature and following a cold challenge. Each of these approaches has limitations, but offer a possible avenue to discover the regulatory pathways involved in cold and febrile induced lysosome biogenesis.

Lipid shuttling during thermoregulation.

Our LIPA deficient model and its cold intolerance questions the current dogma that neutral lipases (ATGL, HSL and MGL) provide free fatty acids necessary for uncoupled mitochondrial thermogenesis. *Atgl-/-* mice are cold-intolerant, similar to *Lipa-/-* mice (Ahmadian et al. 2011). However, our data with adipocyte-specific deletion of *Atgl* and work of others (Zechner) reveal that death of the *Atgl-/-* mice is not due to adipose tissue deficiency but likely to heart failure (Kienesberger et al. 2012). *Atgl-/-* mice suffer from severe cardiomyopathy and typically begin to die around 12 weeks of age. Fat-specific deletion of *Atgl* does not result in a cardiomyopathy (Haemmerle et al. 2006). Cold challenge increases heart rate more than twofold compared to thermoneutrality (Institute of Medicine (US) Committee on Military Nutrition Research, Marriott, and Carlson 1996; Blumberg, Sokoloff, and Kirby 1997). Zechner and colleagues now conclude that "cold intolerance" of *Atgl-/-* mice is likely secondary to heart failure. We did, however, find increased expression of *Lipa* in BAT lacking ATGL. Together, these data suggest that both neutral lipases and LIPA contribute to the pool of fatty acids used for uncoupled oxidation.

Aging.

One of the most common findings in aging is its association with decreased lysosomal and autophagic function. The exact molecular processes engaged are unclear; whether lysosomes directly or indirectly modulate longevity, at least in humans is unknown. Certainly, the current body of evidence provide intriguing

support for lysosomes and longevity. In *C. elegans* data, overexpression of TFEB and a *C. elegans* lipase connected to TFEB, LIPL-4 can both extend lifespan (Lapierre et al. 2011). Studies on caloric restriction and rapamycin have shown that activation of autophagy and reduction of mTOR activity improves longevity. Impaired CMA in the liver promotes aging, while restoration of its function prolongs healthspan (Zhang and Cuervo 2008). These studies point to an obvious relationship between functional autophagy, and consequently, lysosomes with pathological aging.

Similarly, aging is also correlated with impaired homeothermy in both mice and humans (McDonald and Horwitz 1999; Gabaldón, McDonald, and Horwitz 1998; Kerckhoffs et al. 1998). Could lysosomal dysfunction explain the impaired thermoregulation in aged mammalian models? Current hypotheses attribute cold intolerance in aged models to a combination of muscle and BAT atrophy and capacity. In the context of our study, we propose that lysosomes promote thermogenesis, not only through signaling, but also as a direct thermogenic organelle.

Thermoregulation and aging are complex physiological mechanisms. This dissertation provides some potential hints toward the theme of aging. Competent lysosomal activity may contribute a positive thermoregulatory role to whole body physiology. The subsequent metabolic and other physiological responses to proper homeothermy may hence prevent pathological aging.

Therapeutic potential of lysosomal modulation for the treatment of obesity.

Nonshivering thermogenesis research exploded in the last decade in part due to its theoretical potential to increase energy expenditure, independent of lifestyle interventions. Previous attempts to increase energy expenditure pharmacologically resulted in dangerous, non-specific uncoupling of mitochondria and hyperthermia. A more targeted approach of activating thermogenic pathways in relevant cells could avoid such problems. However, the relatively low BAT mass in adult humans makes the substrate for such activation small. This dissertation proposes a new method of inducing thermogenesis that does not depend on uncoupling mitochondria. Further, by exploiting this aspect of biological energy expenditure via lysosomes in both *Ucp1* expressing and negative tissues, low BAT mass limitations in humans is no longer a concern.

Despite some evidence that increasing "browning" of adipose tissue in mice reduce obesity, the success of such an approach in humans assumes that weight regulatory systems will not respond to the increase in energy expenditure. The homeostatic systems that regulate fat mass and body weight "defend" adiposity by mechanisms that are not well delineated; however, these homeostatic systems must somehow not be engaged to increase food intake after "browning."

Regardless of the benefits of weight loss through lysosomal activity, activating a lysosomes directly or indirectly via other areas of the lysosomal pathway may not yield in the anticipated weight reduction. For instance, induction of autophagy without subsequent activation of lysosomal accommodation may result in cellular toxicity. Overexpression of LIPA has additionally lead to disappointing results in the context of atherosclerosis (Zschenker, Illies, and Ameis 2006). It is unclear if LIPA overexpression yields in any other prometabolic markers. Further, if enhancing lysosomal activity, in particular LIPA, increase cellular substrates such

as FFAs, but disregard the necessary simultaneous oxidation and processing of these substrates, cellular toxicity could still occur. In fact, excess FFAs may enhance metabolic dysregulation, exacerbating the very derangement we aim to treat.

If activation of a thermogenic pathway does have therapeutic potential, then activating lysosomal activity may provide a potential new avenue for treating obesity. TFEB, and potentially other transcription factors responsible for both lysosomal biogenesis and catabolism, may be useful pharmacological targets. With its multiple functions within the cell, it would target both the production as well as the processing of lysosomal activity and its subsequent products. A recent study in mice demonstrated that, hepatic TFEB overexpression improves metabolic profiles. Similar prometabolic phenotypes have been found in macrophage specific TFEB overexpression (Emanuel et al. 2014). Such observations not only support the data presented here but also demonstrate the efficacy of TFEB's therapeutic candidacy.

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